Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, 2nd Edition

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Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, 2nd Edition

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A catalogue record for this book is available from the British Library, London, UK

Library of Congress Cataloging-in-Publication Data

Fish diseases and disorders.–2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN-10: 0-85199-015-0 (alk. paper)
ISBN-13: 978-0-85199-015-6 (alk. paper)
1. Fishes–Diseases. 2. Fishes–Infections. I. Woo, P.T.K. II. Title.

SH171.F562 2006
639.3–dc22

2005018533

ISBN-13: 978 1 84593 554 2

Commissioning editor: Rachel Cutts
Production editor: Kate Hill

Typeset by AMA Dataset, Preston, UK.
Printed and bound in the UK by the MPG Books Group.

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Preface to the 1st Edition

This comprehensive volume covers the major viral, bacterial and fungal diseases in fin- and shellfishes. It completes the three-volume series on fish diseases and disorders; Volume I (published in 1995) is on parasitic diseases in fin- and shellfishes while Volume II (published in 1998) deals with non-infectious disorders in finfish. Reviews in the three volumes are written by international authorities that are actively working in the area or have contributed greatly to our understanding of specific piscine diseases or disorders. Authors in the present book are experts on infectious microbial diseases, and are from North America, Europe and Asia.

As in Volumes I and II, the principal audience of this volume is research scientists in the aquaculture industry and universities, fish health consultants and managers of government fish health laboratories. This volume is also appropriate for graduate and senior undergraduate students interested in microbial diseases of fish.

The secondary audience includes parasitologists and environmental toxicologists who may wish to initiate research programmes on the combined effects of microbial and parasitic infections on fish health and the synergistic effects of pollutants on microbial diseases. We expect this secondary audience to increase as it becomes evident that this combined effect can have a great impact on fish health and production and that fish health can also be used as an indicator of problems in the aquatic ecosystem.

We would like to thank Dr R.M.W. Stevenson, Department of Microbiology, University of Guelph, for her advice during the planning of this book.

Patrick T.K. Woo
David W. Bruno

Preface to the 2nd Edition

The second edition of this book represents a major update on the viral, bacterial and oomycete disorders of finfish and shellfish. Since publication of the first edition (in 1999), considerable advances have been made and therefore all the chapters have been thoroughly revised. The new and more eloquent research and current techniques have extended our knowledge and understanding of these infectious organisms. We are pleased that researchers from Europe, North America, Australia and Asia have been involved in updating this book. With the addition of new information, some of the older texts in the original chapters have been condensed; this is to ensure we have focused and comprehensive reviews. For this edition, we have also deleted and/or combined a couple of the original chapters, and have commissioned three new chapters (Chapter 6 on 'Alphaviruses', Chapter 7 on 'Oncogenic Viruses' and Chapter 21 on 'Genomics of Finfish and Shellfish Microbial Pathogens'), which have been written by new authors.

On a more personal note, we are delighted to welcome our 22 new authors who have offered to write new chapters and/or update many of the original chapters. Obviously, we are most grateful to our authors of the first edition for their continued support and contributions to update this volume.

The aims, philosophy, focus, audience and format of this second edition have remained unchanged, and we hope that this edition will continue to be useful to colleagues. Finally, we thank the many CABI personnel, especially Ms Rachel Cutts, for their help and advice during the preparation and production of this book.

Patrick T.K. Woo
David W. Bruno

1 Infectious Pancreatic Necrosis and Associated Aquatic Birnaviruses

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Introduction

Infectious pancreatic necrosis (IPN) is a disease of salmonid fish reared in intensive culture systems. Its aetiological agent (IPNV) was the first virus to be isolated from teleosts (Wolf *et al.*, 1960) and the disease and the virus have been associated intimately with the development of fish and aquatic animal virology. Due to its distinct biophysical characteristics, IPN virus became the archetype of a new family of viruses, the birnaviruses, denoting its bi-segmented RNA genome (Dobos *et al.*, 1979). Over time, numerous closely related viruses of the same family have been detected in a variety of aquatic species, but with diverse clinical manifestations. Thus, the term 'aquatic birnaviruses' has been used to summarize virus family members derived from aquatic animals and environments, while the term 'IPNV' is used for aquatic birnaviruses isolated from or causing disease in salmonid species.

A number of comprehensive reviews have been published on the IPN disease and its causative virus (Wolf, 1966, 1988; McAllister, 1979; Hill, 1982; Reno, 1999). During the past 10 years, research has continued, focusing on molecular markers of virulence, host–pathogen relationships, epidemiology, on the role of inducing factors in disease manifestation and on the development of new and more effective methods for its control in commercial salmonid farming. Major progress has been made in vaccination against IPN, in selective breeding and in the search for genomic markers of resistance to IPN in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Without repeating earlier reviews, we will try to integrate the more recent findings on IPN and IPNV with earlier knowledge and provide a relevant update on the disease and the virus.

Clinical Signs of the Disease

The disease was first described as acute catarrhal enteritis of brook trout (*Salvelinus fontinalis*) fry by McGonigle (1940) and was for many years thought to affect only hatchery-raised fry. Disease outbreaks were noted on the east coast of America in brook trout and rainbow trout fingerlings immediately after first feeding commenced (Wood *et al.*, 1955; Snieszko *et al.*, 1957) and its infectious nature was soon established (Snieszko *et al.*, 1959). Mortality in young fry with IPN has been reported at over 90% (Vestergård-Jørgensen and Bregnballe, 1969) and, although the individual value of fry is low, acute IPN of feeding fry and fingerlings remains an important cause of losses

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Viral, Bacterial and Fungal Infections, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

in salmonid aquaculture. It was not until the mid-1980s that Norwegian fish health workers first reported disease outbreaks in farmed Atlantic salmon post-smolts (Christie *et al.*, 1988) and this new manifestation soon arrived in Scotland (Smail *et al.*, 1992). Either the farmed Atlantic salmon carry the virus prior to seawater transfer and disease is being induced by the change in environmental conditions or the fish become infected in the sea by horizontal transmission. In IPN post-smolts, the onset of disease occurs typically 4–12 weeks post-transfer of the fish (Brun, 2003).

There are no specific pathognomonic signs of IPN disease. However, a combination of behavioural changes and gross internal and microscopic lesions can be used to form a tentative IPN diagnosis. In salmonid fry, the behavioural signs include anorexia and a violent whirling swimming motion (Wood *et al.*, 1955) or swimming on the side. Non-specific external signs include darkening of the skin, abdominal swelling (Fig. 1.1), exophthalmia, pale gills and petechial haemorrhages on the ventral surface, including fins. Many fish also trail thin white/yellow casts from their anal orifice (Wolf, 1988). There is a lack of food in the digestive tract, but it may contain a milky mucous substance and there are often petechial haemorrhaging and visceral ascites. In some individuals, the spleen, heart, liver and kidney are abnormally pale (Wolf, 1988). The incubation period is normally 4–7 days in brook trout or rainbow trout fry and 10–14 days in Atlantic salmon fry (Okamoto *et al.*, 1993; Taksdal *et al.*, 1997; Wetten *et al.*, 2007).

In Atlantic salmon post-smolts, IPN manifests through a loss of appetite and increased mortality, sometimes preceded by aberrant swimming behaviour, but mostly without marked external signs. There are usually an empty stomach and intestine with catarrhal, viscous exudate and pale yellow liver (Roberts and Pearson, 2005). Petechial haemorrhage may be seen in the pyloric fat tissue. Following outbreaks, a fraction of the survivors may become cachectic and eventually succumb (Smail *et al.*, 1992). The incubation period of clinical disease in Atlantic salmon post-smolts is normally between 10 and 14 days at 10°C (Bowden *et al.*, 2002; Ramstad *et al.*, 2007).

IPN exhibits marked pancreatic necrosis and severe lesions in the intestinal mucosa. Pancreatic lesions can vary from small foci to extensive acinar cell necrosis (Fig. 1.2), with nuclear pyknosis, karyorrhexis and basophilic cytoplasmic inclusions (Wolf, 1988; Smail *et al.*, 1992). The acute effects on the gut mucosa were detailed in rainbow trout by McKnight and Roberts



Fig. 1.1. Infectious pancreatic necrosis disease in rainbow trout fry displaying abdominal swelling. Image supplied by Dr Torunn Taksdal (original), National Veterinary Institute, Norway.

(1976), who described acute sloughing of the mucosal epithelium, which was also reported later in IPNV infected Atlantic salmon post-smolts (Smail *et al.*, 1995). Congestion and necrosis of liver tissue are marked findings (Taksdal *et al.*, 1997; Roberts and Pearson, 2005). In both fry and post-smolts, the disease may be manifested with all, some or no clinical signs.

Geographical Distribution and Host Range

The birnaviruses infect numerous aquatic animal species and are found in all continents of the globe; consequently, they have been rightfully designated 'the most pervasive pathogens of aquatic animals' by Reno (1999). He produced a detailed account of their host species range and geographical distribution, and information published during the last decade has supplemented and confirmed his review.

Surveys in eastern as well as western parts of the North Atlantic have demonstrated that birnaviruses are relatively common in marine wild fish populations, especially in various flatfish species (Table 1.1). Danish surveys yielded 45 isolates from the North Sea and the adjacent seas between Denmark and Norway (Skagerrak) and Denmark and Sweden (Kattegat), all belonging to serogroup B and thereby clearly distinct from the bulk of salmonid isolates (Skáll *et al.*, 2000). No aquabirnavirus was found, however, in 280 pooled samples obtained in the Baltic Sea. In another survey carried out by Spanish researchers, birnavirus isolates were obtained from several marine species caught in the Flemish Cap, off the coast of Newfoundland. The isolates apparently were not

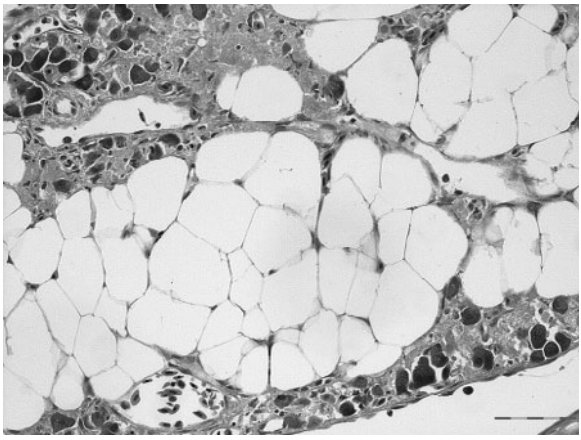


Fig. 1.2. Typical pancreatic acinar cell necrosis due to IPNV infection. Bar scale=10 µm. Image supplied by Patricia Noguera (original), Marine Scotland, Scotland.

Table 1.1. Isolation of birnaviruses from wild fish species caught in oceanic surveys.

Geographical location	Fish	Reference
North Sea, Skagerrak, Kattegat	Atlantic cod, European plaice, grey gurnard, long rough dab, smear dab, flounder	Skáll *et al.* (2000)
Flemish Cap	Atlantic cod, Greenland halibut, Atlantic wolf-fish, onion-eye grenadier, deepwater redfish	Romero-Brey *et al.* (2004)

serotyped, but genotyping based on parts of the VP2 gene suggested that the isolates were related most closely to strains (West Buxton, Dry Mills) that belonged to serotype A1 (Romero-Brey *et al.*, 2004).

In light of these findings, the historical emergence of IPN in north-eastern America and in Europe, respectively, may not have been pure coincidence but a consequence of the anadromous life cycle of salmonids combined with the growth and intensification of salmonid culture. The relatively frequent occurrence of birnavirus infection and disease in eel (*Anguilla anguilla)* (Vestergård-Jørgensen *et al.*, 1994; Haenen *et al.*, 2001; van Ginneken *et al.*, 2004) may serve to support this hypothesis, as the farming of eel in Europe is based on elvers (juvenile seed-stocks) caught during their anadromous migration from the Atlantic Ocean. Isolation of birnaviruses from two marine species (sand eel [*Ammodytes* sp.] and blue whiting [*Micromesistius poutassou*]) used for preparation of feeds for farmed turbot (*Psetta maxima*) (Rivas *et al.*, 1993) adds weight to this hypothesis.

There are no similar wild fish surveys from other oceanic areas, but a significant number of scientific reports from Japanese and Korean mariculture suggest that a second 'hot spot' for aquatic birnaviruses exists in Japanese coastal areas and the Sea of Japan bordering the East China Sea. Kusuda *et al.* (1993) analysed Japanese birnavirus isolates from several marine species, Suzuki *et al.* (1998) found the virus in pearl oysters (*Pinctada fucata*) and Jung *et al.* (1999) in cultured ayu (*Plecoglossus altivetis*). Isshiki *et al.* (2004) reported that, among 1291 pooled tissue samples from a diversity of marine fish species cultured in Kawaga prefecturate in Japan, birnaviruses were detected in yellowtail (*Seriola quinqueradiata*), amberjack (*S. dumerili*) and Japanese flounder (*Paralichthys olivaceus*) using cell culture, and additionally in spotted halibut (*Verasper variegatus*) and goldstriped amberjack (*S. lalandi*) using PCR. In Korea, birnavirus isolations were reported from cultured flounder (Sohn *et al.*, 1995), rockfish (*Sebastes schlegeli*) (Seo and Heo, 1998) and frequently, over many years, from cultured Japanese flounder (Jung *et al.*, 2008). Considering these reports, the known host range for aquatic birnaviruses has likely now exceeded 100 species.

There have been no major changes in the geographical distribution of IPNV or aquatic birnaviruses during the past decade. A single detection has been reported in Chinook salmon (*O. tshawytscha*) returning to New Zealand (Tisdall and Phipps, 1987) and there is one report of birnavirus detection in marine-cultured salmonids as well as in local wild fish species in western Tasmania (Crane *et al.*, 2000). First reports of IPNV in farmed rainbow trout have been made from Turkey (Candan, 2002), Greece (Varvarigos and Way, 2002) and Mexico (Ortega *et al.*, 2002). IPN was first reported by Pitchugina *et al.* (2003) in Atlantic salmon reared in the Russian Murmansk region. Based on intensive surveillance documentation, Swedish salmonid aquaculture is considered free of IPNV (Ariel and Olesen, 2002). In mainland Australia and Iceland, it appears that the virus has not been found, despite intensive fish virus surveillance over many years. In most wild fish populations including wild salmonids, the prevalence of birnaviruses remains low and detection is only occasional and highly dependent on the intensity of monitoring and surveillance efforts. Their worldwide geographic distribution, however, serves to illustrate that there is a latent risk that clinical disease including IPN may emerge from unknown aquatic birnavirus reservoirs and spread within aquaculture production systems unless effectively monitored and controlled.

Regions with frequent isolations of IPNV (Norway, Scotland, Ireland, eastern North America, Chile, parts of Continental Europe) or of other aquatic birnaviruses (Japan, Korea, Spain) are characterized by intensive farming of Atlantic salmon, rainbow trout or marine flatfish species. It is therefore tempting to hypothesize that the geographical distribution of birnaviruses including IPNV is mainly a reflection of aquaculture production and its population structure and dispersion, and less a question of the spatial presence of the virus in the first place.

Specified clinical diseases in non-salmonid species caused by aquatic birnaviruses are relatively few and have been found mainly in eel and flatfish species (Table 1.2).

Among the described pathologies are several disease conditions of eel caused by an aquatic birnavirus named eel virus European (EVE; Sano *et al*., 1981). Birnavirus isolates derived from turbot and Atlantic halibut (*Hippoglossus hippoglossus*) have been used experimentally to induce clinical disease in the homologous species (Mortensen *et al*., 1990; Biering, 1994) but failed in scallops (*Pecten maximus*) (Mortensen *et al*., 1992).

In the vast majority of cases, non-salmonid birnavirus isolates have failed to produce disease in salmonids (Reno, 1999) and it appears that this conclusion still holds true. However, Rivas *et al*. (1993) reported that birnavirus isolates obtained from mussels (*Mytilus galloprovincialis*) were pathogenic to rainbow trout. Also, Perez-Prieto *et al*. (2001) were able to induce mortality experimentally in rainbow trout and sea bream (*Sparus aurata*), but not in brown trout using a birnavirus isolate from Senegalese sole (*Solea senegalensis*). Rodger *et al*. (1996) infected brown trout with an IPNV isolate from sea bream and were able to induce pancreatic necrosis in recipient fish, but apparently without mortality.

Due to the high prevalence of IPN virus in industrial salmonid farming, there has been a growing interest in assessing persistence and pathogenicity of IPNV in prospective and emerging aquaculture species, including species that, if becoming covertly infected, may serve as reservoirs or vectors in the epizootiology of IPN (Table 1.3).

Using IPNV isolates that had previously proven virulent in Atlantic salmon and Atlantic halibut, respectively, Sommer *et al*. (2004) were able to induce clinical disease

Table 1.2. Disease conditions in non-salmonid species caused by aquabirnaviruses.

Fish	Scientific name	Disease name	Reference
Atlantic menhaden	*Brevoortia tyrannus*	'Spinning disease'	Stephens *et al*. (1980)
Japanese eel	*Anguilla japonica*	Eel branchionephritis Gill lamellar pillar cell necrosis	Sano *et al*. (1981); Lee *et al*. (1999)
European eel	*Anguilla anguilla*	Unnamed	Haenen *et al*. (2001)
Yellowtail	*Seriola quinqueradiata*	Yellowtail ascites disease	Sorimachi and Hara (1985)
Turbot	*Psetta maxima*	'IPN'	Castric *et al*. (1987); Mortensen *et al*. (1990)
Atlantic halibut	*Hippoglossus hippoglossus*	'IPN'	Mortensen *et al*. (1990); Biering (1994)
Senegalese sole	*Solea senegalensis*	Solevirus disease	Rodriguez Saint-Jean *et al*. (1997)

Table 1.3. Studies involving experimental challenge of prospective fish farming species with IPNV.

Fish	Challenge isolate from	Mortality	Reference
Spotted wolf-fish	Atlantic salmon, Atlantic halibut	Up to 67% in juveniles	Sommer *et al*. (2004)
Atlantic cod	Atlantic salmon	17–20% in juveniles	Urquhart *et al*. (2009)
Atlantic cod	Atlantic halibut	None	Jensen *et al*. (2009)
Noble crayfish	Rainbow trout	None	Halder and Ahne (1988)

with up to 67% mortality in groups of juvenile spotted wolf-fish (*Anarhichas minor*) (<0.3 g), challenged via the water, whereas larger juveniles (0.7 g) remained healthy. The virus persisted for >4 months in survivors of these experiments. Scottish workers (Urquhart *et al.*, 2009) were recently able to induce up to 20% mortality in 1–3 g juveniles of Atlantic cod (*Gadus morhua*) after waterborne infection using an IPNV isolate (serogroup A2) from an outbreak in Atlantic salmon. Larger fish (10 g) that became infected without clinical signs were shown to carry the virus in macrophages for up to 12 weeks (Garcia *et al.*, 2006). A Norwegian study with 40 g cod juveniles also failed to induce disease through experimental infection, but IPN virus persisted in the fish for 6 weeks (Jensen *et al.*, 2009). Salmonid IPNV was shown to persist in freshwater crayfish (*Astacus astacus*) for up to 1 year (Halder and Ahne, 1988), even though there was neither mortality nor a definite proof of viral replication in the organs of the crayfish.

In summary, the exceptionally wide geographical distribution and host range of aquatic birnaviruses point towards their potential to cause new disease in most emerging fish farming species and to threaten the development of polyculture systems where zoo-sanitary controls become complex and impracticable.

Economic Importance of the Disease

The economic impacts caused by IPN are both direct and indirect, due to clinical disease and mortality and the costs of preventive and control measures such as health inspections, viral surveillance, diagnostic investigations and, last but not least, the destruction of infected but healthy stocks due to legal provisions. Reno (1999) quoted several examples from North America where attempts to clear hatcheries from IPNV led to the destruction of up to 7 million salmonid eggs or juveniles and argued that the statutory slaughter of infected fish and movement restrictions might be more economically detrimental than the loss associated with direct mortalities. This has rendered zoo-sanitary control strategies for IPN subject to controversy and, in most countries with a significant commercial salmonid farming industry, the regulation of IPNV infection has been relaxed. Where clinical disease particularly affects fry and juveniles only, the losses can be ameliorated easily by spawning additional broodfish to compensate for potential mortality (Reno, 1999).

With the rapid growth of Atlantic salmon farming, and in particular the increasing impact of IPN outbreaks following sea transfer (Jarp *et al.*, 1994), new attention was drawn towards the economic impact of IPN. At this stage of development, the disease affects individuals between 9 and 18 months of age, weighing from 40 to above 100 g and, consequently, a direct loss of around €1–1.5 (US$1.5–2.5) apiece or 8–10 times higher than the loss of fry. Secondly, does the compensatory smolt production face logistical limitations (tank space and time), making it a less attractive or sometimes non-existent alternative? Adding to the direct losses caused by post-smolt IPN outbreaks are the impaired growth and performance of a fraction of the surviving fish (Smail *et al.*, 1992). The growing economic impact of clinical IPN in Norwegian and Scottish Atlantic salmon farming therefore stimulated parallel efforts to reassess the situation and the national control policies for this disease from 2000 to 2003 (Anon., 2003; Skjelstad *et al.*, 2003).

It is surprising there are no publications in scientific journals to provide quality estimates of direct and indirect losses due to IPN in salmon farming, or to assess the benefit–cost ratio of various measures to control the disease or the infection. A number of Norwegian studies, however, have addressed important elements of the animal health economics of IPN and have provided some background information for economic assessment. The most important consequence in commercial salmon farming is perceived to be due to IPN-specific post-smolt mortality, which is estimated between 4.5 and 12% of the total number of smolts put to sea (Table 1.4).

Table 1.4. Estimation of IPN-specific losses among Atlantic salmon post-smolts, based on differences in cumulative mortality observed during Norwegian epidemiological studies.

Year put to sea	1991	1993	1998	1999	2000	2001	2002
IPN outbreak (%)	17.8	9.0	17.8	12.6	10.3	10.9	13.3
No IPN outbreak (%)	11.4	1.8	5.8	5.5	5.8	5.3	6.7
Difference (%)	6.4	7.2	12.0	7.1	4.5	5.6	6.6
Reference	Jarp *et al.* (1994)	Jarp and Melby (1997)	Lervik *et al.* (2003)	Lervik *et al.* (2003)	Lervik *et al.* (2003)	Lervik *et al.* (2003)	Lervik *et al.* (2003)

Considering the increase in Norwegian smolt output from about 90 million (1993) to 140 million (2002), these figures correspond to losses of 5–10 million smolts/year, which at a conservative price estimate of €1 apiece equals the same direct loss in euro.

In an IPN survey of the 2001 smolt output year class carried out by the Shetland Salmon Farmers Association (UK), the IPN-specific losses were estimated at 10% (1.4 million fish) of the spring smolt output, while 16% IPN-specific losses were reported among the 2000 S1/2 fish that had been transferred to sea the previous autumn (Anon., 2003, Appendix V). According to the General Manager of the Association, the corresponding economic loss amounted to GB£2 million (*Fish Farmer*, November/December 2001, pp. 8–10).

Economic estimates presented at conferences and 'unpublished' data suggest that the combined losses (fry and juveniles plus post-smolts) in the Norwegian salmon farming industry exceeded €12 million in 1998 (Lars Liabø, Kontali Analyse AS, cited after handouts from a conference organized by the Norwegian Fish Farmers' Association, 20 November 1998). Relating to 2001 data, the combined effect of fry and post-smolt losses was estimated at €7.4 million (Asgeir Østvik, DVM; proceedings from a seminar organized by the Norwegian Veterinary Association, October 2002). In a questionnaire survey carried out among Norwegian fish farm managers and fish health specialists, both hatchery managers and marine site managers ranked the protracted health effects of an IPN outbreak (pinheads, impaired growth, 'louse catchers') of equal importance as direct mortality, with negative operational consequences for production (extra workload, unpredictability of logistics and planning) following closely (Aunsmo *et al.*, 2003). This indicates that the indirect economic consequences of IPN as seen currently in commercial salmon farming are considerably higher than the costs of the mortality itself.

Infectious Pancreatic Necrosis Virus (IPNV)

The agent

There are several comprehensive reviews on the biophysical and biochemical characteristics of IPNV and aquatic birnaviruses (Wolf, 1988; Dobos, 1995a; Rodriguez Saint-Jean *et al.*, 2003; Evensen and Santi, 2008). Consequently, the emphasis in the present discussion will be on the characteristics that may play a role in the disease process and epizootiology.

Taxonomic Classification

Infectious pancreatic necrosis virus is the type species of the genus *Aquabirnavirus* within the viral family designated *Birnaviridae* by the International Committee on Taxonomy of Viruses (Delmas *et al.*, 2005). Other genera within this family are *Avibirnavirus* and *Entomobirnavirus*, which infect

birds and insects, respectively. Aquatic birnaviruses make up the largest group of viruses within the *Birnaviridae* and have been isolated from a large number of fish, molluscs and crustaceans. The term 'infectious pancreatic necrosis virus' has been used inconsistently and, for stringency, we will refer to IPNV when an aquatic birnavirus has been isolated from salmonids or when causing disease in salmonids. All other IPNV-like viruses are detailed as aquatic birnaviruses.

Physiochemical Properties

The IPN virion is non-enveloped, single shelled (Lightner and Post, 1969) and measures approximately 60 nm in diameter (Cohen *et al.*, 1973). Dobos *et al.* (1977) determined that the virion had a buoyant density in caesium chloride of 1.33 g/cm^3, a sedimentation coefficient of 435S and a total molecular weight of 55×10^6 Da, and calculated that the weight of the capsid protein was approximately 50.2×10^6 Da with an RNA component of 4.8×10^6 Da.

IPNV is lyophilized easily in the presence of skimmed milk, lactose or lactalbumin hydrolysate (Wolf *et al.*, 1969). Gosting and Gould (1981) found that IPNV cultured in cell culture medium buffered to pH 7.2 was relatively thermal stable, and Mortensen *et al.* (1998) reported that IPNV was stable at a variety of salinities ranging from 0 to 40% and that, in water, temperature only influenced stability when above 20°C. The infectivity of IPNV persists for 5–6 months in filter-decontaminated water at 4°C (Baudouy and Castric, 1977). Ahne (1982) documented that IPNV infectivity persisted for over 7 months in bacteria-free tap water held at 10°C, but in non-treated canal water IPNV was not detectable after 14 days. Both Toranzo and Hetrick (1982) and Barja *et al.* (1983) revealed that IPNV exhibited the greatest stability in estuarine water held at 15°C, where the time required for a 3-$\log_{10}$ reduction in infectivity was 27 days. They also reported that the lowest viability of IPNV was recorded from fresh water held at 20°C, where a 3-$\log_{10}$ reduction in infectivity was found after only 9 days. Normal seawater temperatures do not seem to affect IPNV survival significantly. IPNV infectivity persisted nearly four times longer in filter-sterilized or autoclaved estuarine water compared with untreated estuarine water, suggesting that microbial flora played an important role in degrading IPNV (Toranzo *et al.*, 1983).

IPNV infectivity decreases during a freeze/thaw process; however, the tolerance of differing isolates varies greatly (McMichael *et al.*, 1975). Wolf and Quimby (1971) reported that four French isolates lost at least 99.9% of their infectivity during one freeze (–20°C for 1 h)/thaw sequence. The authors also detailed that the four isolates were stable in 50% glycerol at 4°C and that three of the isolates remained viable for 4 years. Mortensen *et al.* (1998) studied the effect that repeated freezing and thawing had on an IPNV Sp isolate and demonstrated that the reduction in viral titre was much greater in virus stored at –80°C compared with virus stored at –20°C. They also reported that the viral titres obtained from kidney tissue sampled from IPNV-carrier Atlantic salmon decreased by at least a factor of 10 within 24 h and by a factor of 100 after 48 h at 5°C. They hypothesized that this reduction in infectivity might be due to virus-inactivating factors such as neutralizing antibodies produced by the host or tissue enzymes. This is a very important finding as it is common for diagnostic laboratories to store tissue homogenates overnight at 4°C before inoculation on to cell cultures.

Serological Classification

A comprehensive update of the serological characterizations of aquatic birnaviruses was provided by Reno (1999), who emphasized that the serological classification of aquatic birnaviruses served multiple purposes, including understanding epizootiological studies and vaccine development.

It was evident to early researchers that distinct serotypes of aquatic birnaviruses existed, as rabbit antibodies produced

against the type strain VR-299 only partially neutralized two European isolates (Wolf and Quimby, 1971). Hill and Way (1995) suggested a standard serological classification scheme for aquatic birnaviruses, which was accepted universally by fish health professionals. They classified the aquatic birnaviruses into two serogroups (A and B) by performing cross-neutralization assays using polyclonal antisera on nearly 200 aquatic birnavirus isolates. No cross-reactivity between the two serogroups was noted. They divided serogroup A isolates into nine serotypes, termed A1–A9, based on the reciprocal cross-neutralization, and assigned historical type strains to the new system (Table 1.5). However, there is a variable degree of antigenic cross-reactions between the nine serotypes within serogroup A. All of these archetypal strains within serogroup A originated from Europe or North America and were associated with IPN disease in salmonids, with the exception of serotype A4 (He strain) isolated from pike (*Esox lucius*) and serotype A5 (Te) isolated from a mollusc (*Tellina tenuis*). However, both the A4 and A5 serotypes have subsequently been isolated from salmonids (Reno, 1999). To summarize, the majority of isolates originating from the USA belong to serotype A1; A2–A5 strains have been isolated predominantly from Europe; with serotypes A6–A9 from Canada. Salmonid isolates from Asia and South America (Espinoza *et al.*, 1985; Ortega *et al.*, 2002) tend to fall into serotypes A1–A3, most probably being introductions from Europe and North America (Espinoza *et al.*, 1985). In Norwegian and Scottish Atlantic salmon aquaculture, the vast majority of aquatic birnaviruses has been classified as A2 serotype (Krogsrud *et al.*, 1989; Melby *et al.*, 1994; Smail *et al.*, 2006).

Serogroup B contains only one serotype, but this is based on a limited number of isolates. However, some isolates have been found to be pathogenic to salmonids (Ahne *et al.*, 1989), while others have been termed avirulent (Olesen *et al.*, 1988). Danish researchers isolated 45 birnavirus serogroup B isolates during a cruise survey of more than 17,000 wild marine fish in the waters surrounding Denmark (Skáll *et al.*, 2000). They reported that 43/45 serogroup B isolates originated from wild flatfish species including plaice (*Pleuronectes platessa*), dab (*Limanda limanda*), flounder (*Platichthys flesus*), smear dab (*Microstomus kitt*) and long rough dab (*Hippoglossoides*

Table 1.5. Grouping of IPNV and aquatic birnavirus isolates based on *in vitro* seroneutralization (Hill and Way, 1995) and genomic analysis (Blake *et al.*, 2001).

Proposed serotype nomenclature (Hill and Way, 1995)	Origin	Historical serotype nomenclature	Proposed genogroup (Blake *et al.*, 2001)
		Serogroup A	
A_1	USA	WB (West Buxton/VR299)	1
A_2	Denmark	Sp (Spjarup)	5
A_3	Denmark	Ab (Abildt)	2
A_4	West Germany	He (Hecht)	6
A_5	UK	Te (Tellina)	3
A_6	Canada	Can. 1	3
A_7	Canada	Can. 2	4
A_8	Canada	Can. 3	4
A_9	Canada	Ja (Jasper)	1
		Serogroup B	
B_1	UK	TV-1	N.d.

N.d., not determined.

platessoides). 'Hot spot' areas for birnavirus serogroup B isolates appeared in the North Sea, Skagerrak and Kattegat areas. The authors also mention that birnavirus serogroup B has been isolated only twice from healthy farmed rainbow trout over a 30-year period in Denmark.

Several scientists have developed monoclonal antibodies (MAbs) to assist with the classification of aquatic birnaviruses (Caswell-Reno *et al.*, 1986, 1989; Christie *et al.*, 1988). Although, in general, these studies agreed with the serotypes categorized using polyclonal antisera neutralization assays, there have been studies using MAbs (in immunoblot or ELISA assays) that could not discriminate between all nine serotypes within serogroup A (Christie *et al.*, 1990; Melby and Christie, 1994; Frost *et al.*, 1995). Therefore, there is currently no suitable assay using a single or low number of MAbs to determine the serotype of a particular aquatic birnavirus.

Viral Genome

Dobos (1995a) conducted a detailed review into the IPNV genomic structure and function. The viral genome consists of a bi-segmented, double-stranded RNA which represents approximately 9% of the total virion weight. The viral capsid is thought to be built up of two structural proteins, designated viral protein (VP)2 and VP3. These two structural proteins are coded for by segment A, which also codes for two additional non-structural (NS) proteins, the protease (VP4) and a protein whose full function is unknown (VP5). Segment B encodes only one protein, an internal polypeptide designated VP1.

The largest part of the genome is segment A, which comprises between 2962 and 3097 bp. It contains two open reading frames (ORFs): one large ORF encoding a 106 kDa polyprotein that is co-translationally cleaved by the virus encoded serine-lysine protease (VP4) to produce two polypeptides, pVP2 (63 kDa, a precursor of VP2) and VP3 (31 kDa), a minor capsid protein (Duncan *et al.*, 1987; Dobos, 1995a). The pVP2 protein is further cleaved during virus maturation to the major capsid protein, VP2 (54 kDa) (Dobos, 1995a; Galloux *et al.*, 2004). The coding order of the virus proteins in IPNV is NH2–pVP2–VP4–VP3–COOH. Segment A also contains a small ORF, which overlaps the 5′-terminal of the large ORF and is in a separate reading frame (Duncan *et al.*, 1987). This smaller ORF encodes an arginine-rich minor nonstructural protein, VP5 (15 kDa) (Magyar and Dobos, 1994). The smaller genome segment B (~2900 bp) is monocistronic and encodes the VP1 protein (94 kDa), which functions as the viral RNA-dependent RNA polymerase (RdRp) (Duncan *et al.*, 1991; Dobos, 1995b).

Molecular Classification

Due to the limitations experienced during serotyping of aquatic birnaviruses, it was important for scientists to investigate and develop new techniques to characterize viral isolates. Several techniques were used in the early stages of the molecular analysis of aquatic birnaviruses: nucleotide and deduced amino acid sequence analysis (Heppell *et al.*, 1993), RNA fingerprinting (Hsu *et al.*, 1995) and restriction fragment length polymorphism (RFLP) profiles of the VP2 gene (Lee *et al.*, 1996; Biering *et al.*, 1997). The former method – comparison of the nucleotide and deduced amino acid sequences – has become the most extensively used technique for comparison and genetic classification of fish viral isolates. As detailed previously, the VP2 is the major capsid protein and contains the neutralization epitopes. For this reason, most molecular virologists have chosen to focus their research on the VP2 coding region. However, it is worth noting that Romero-Brey *et al.* (2009) sequenced the whole genome of seven aquatic birnavirus strains isolated from several wild fish species and reported one isolate to cluster as WB type from the analysis of genomic segment A, but as type Ab from analysis of genomic segment B. This finding suggests that natural reassortment of two differing aquatic birnavirus

strains may take place in a host, and the authors have advised that both genomic segments should be sequenced to better characterize birnavirus isolates.

A study examining the phylogenetic relationships of aquatic birnaviruses based on the nucleotide and deduced amino acid sequences of the large ORF of segment A of the nine type strains of serogroup A and the VP2 gene of several other isolates within serogroup A was conducted by Blake *et al.* (2001). The results suggested that the nine strains of serogroup A would fall into six genogroups based on phylogenetic analysis of the VP2 gene on 28 aquatic birnavirus isolates (Table 1.5). According to this system, genogroup 1 would comprise the US isolates, including the West Buxton (serogroup A1) type strain, and two Jasper strains from Canada. It was also proposed that genogroup 1 contained at least four different genotypes. Genogroup 2 consists of isolates from Asia and Europe (A3). Genogroup 3 consists of two Canadian isolates (C1 and ASV) (A6), as well as the European Te isolate (A5). Genogroup 4 contains the two Canadian isolates, C2 and C3, representing serotypes A7 and A8, respectively. Genogroup 5 contains five European isolates, including the type strain Sp and one Asian isolate (A2), and genogroup 6 consists of the He strain (A4). The authors went on to explain that the isolates representing the three major serotypes in Canada were related to the European isolates more closely than those from the USA and that, in general, the genogroups correlated to previous serological classifications and geographical origin. Since this study, several other scientists have characterized aquatic birnaviruses at the genetic level (Cutrín *et al.*, 2004; Zhang and Suzuki, 2004; Bain *et al.*, 2008; Ruane *et al.*, 2009a). A seventh genogroup comprising Japanese aquatic birnaviruses originating from marine fish was proposed by Nishizawa *et al.* (2005) after sequence analysis of 310 base pairs at the VP2/VP4 junction region (Fig. 1.3). Unfortunately, several authors have numbered the genogroups differently from that proposed by Blake *et al.* (2001), which may lead to confusion.

We hope the classification system proposed by Blake *et al.* (2001), supplemented by a seventh genogroup as reported by Nishizawa *et al.* (2005), can be adopted with a universal nomenclature. As the number of IPNV isolates hitherto subjected to genomic analysis is limited, revisions will likely be required as information accumulates. To what degree the genotyping system can provide a general association with serogrouping based on polyclonal or monoclonal antibodies will also be of great interest.

VP1 – the segment B encoded protein

The VP1 protein initiates viral RNA synthesis and originates from translation of RNA segment B. Only the VP1 protein is encoded from genomic segment B and is often termed the RNA-dependent RNA polymerase (RdRp). All RNA viruses must have the ability to translate an RdRp to perform multiple duties in the replication process. Dobos (1995b) determined that VP1 was present in infectious virions and Mertens *et al.* (1982) demonstrated that the optimum enzymatic activity for the IPNV RdRp was achieved at pH 8.0, 30°C and in the presence of 6 mM Mg^{2+}. It was also demonstrated that the IPNV RdRp was active without proteolytic pretreatment of the virus, indicating that uncoating may not be required for virus replication (Cohen, 1975).

VP1 is present in the virion as both a free polypeptide and covalently linked to the 5′ end of the two genomic segments by a serine-5′ GMP phosphodiester bond, and is termed VPg (Calvert *et al.*, 1991). It was later discovered that VP1 can be guanylylated *in vitro* and that this VP1–pGpG complex becomes a primer for *in vitro* RNA synthesis (Dobos, 1995b). Magyar *et al.* (1998) reported that, in IPNV infected cells, VP1 attached to the 5′ terminal sequences of genomic segments A and B positive strands. Pulse-chase experiments in combination with other techniques revealed that VP1 could be chased via replicative intermediates; to single-stranded RNA, to double-stranded RNA and ultimately to virion VPg-dsRNA.

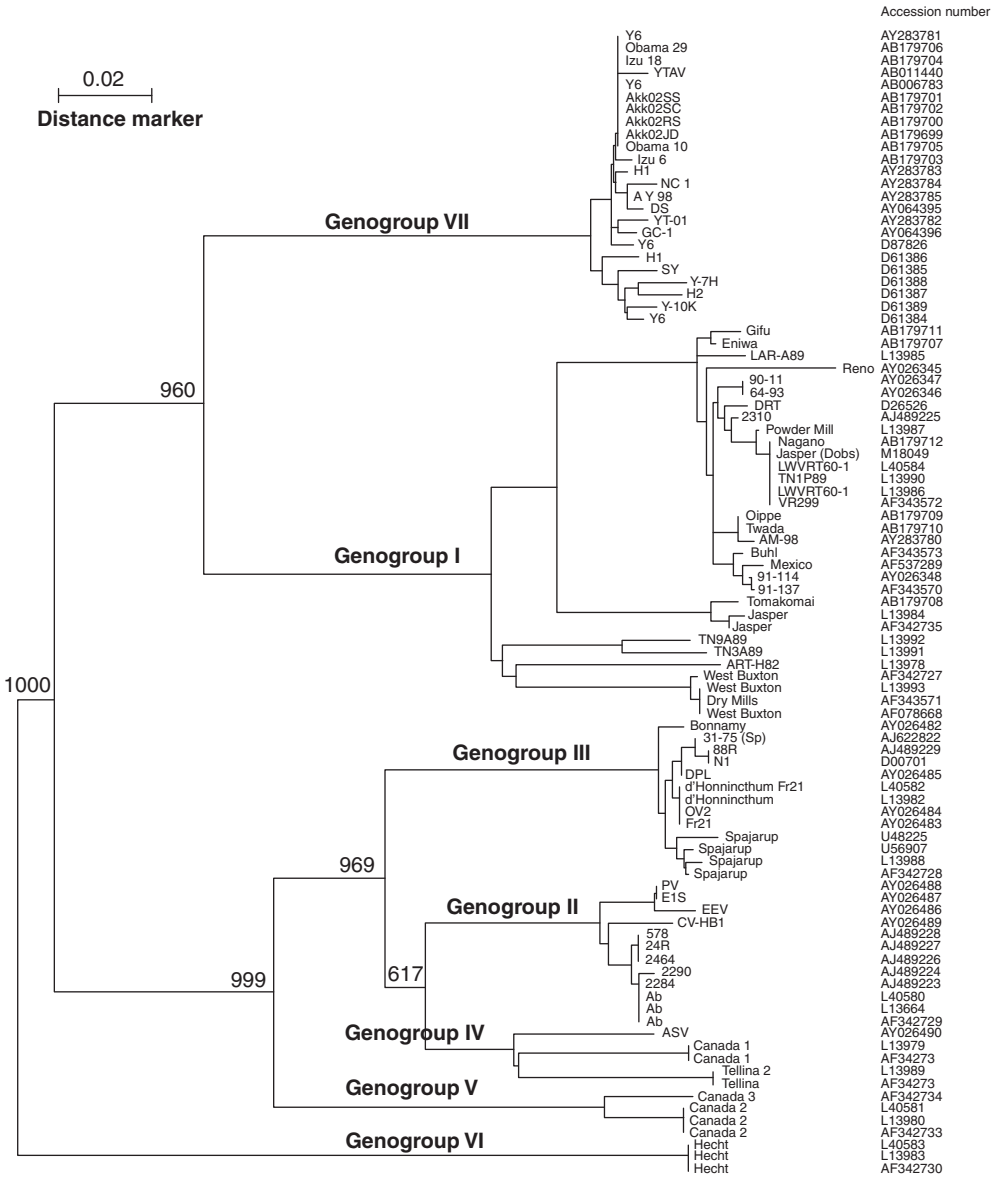


Fig. 1.3. Molecular phylogenetic tree based on nucleotide sequences of the VP2/NS junction among 93 worldwide isolates of IPNV and other aquabirnaviruses, and corresponding Genbank/DDBJ accession numbers. Bootstrap values from 100 replicates are shown at major nodes. Scale: 0.02 replacement nucleotides per site. Reproduced from Nishizawa *et al*. (2005). An approach for genogrouping of Japanese isolates of aquabirnaviuses in a new genogroup, VII, based on the VP2/NS junction region. *Journal of General Virology* 86, 1973–1978, with permission from the Society for General Microbiology.

They also demonstrated that VP1 could be released from the aforementioned structures by the addition of RNase A. These results suggest that a VP1–pG complex acts as a primer for viral plus RNA synthesis *in vivo*. The RdRps of birnaviruses are therefore designed to act as a primer during RNA transcription, as well as a functional polymerase. It is important to note that the IPNV VP1 is extremely large in contrast to the VPgs of other RNA viruses and that birnaviruses are the only dsRNA viruses that contain a VPg. It has also been reported that the VP1 protein of birnaviruses forms a distinct subgroup of RdRps, which do not contain the highly conserved Gly-Asp-Asp (GDD) motif, a characteristic of RdRps from single-stranded plus RNA viruses (Duncan *et al.*, 1991; Shwed *et al.*, 2002). Evidence that the VP1 polypeptide is the IPNV RdRp has been provided by Villanueva *et al.* (2005). They demonstrated that dialdehyde–nucleotide analogues, used to identify nucleotide binding proteins, covalently inhibited IPNV *in vitro* RNA synthesis. They went on to conclude that VP1 was the IPNV RdRp, since it was identified as being bound to dialdehyde–nucleotide analogues, which resulted in the inhibition of the transcriptional activity of the viral enzyme.

Sequence analysis of the VP1 protein of infectious bursal disease virus (IBDV), the type species of the genus *Avibirnavirus*, revealed that strains classified as very virulent were phylogenetically distinct from all other IBDV strains (Islam *et al.*, 2001), which would suggest a role in virulence, and Liu and Vakharia (2004) demonstrated that the VP1 protein modulated the virulence of IBDV *in vivo*. However, Song *et al.* (2005) concluded that VP1 was not involved in the virulence of IPN virus. They created a reassortant virus containing the VP1 protein of a moderately virulent strain (Sp103) and the polyprotein of a highly virulent strain (rNVI15-15KA). An aquaria challenge on Atlantic salmon fry was conducted and they reported that the mean cumulative mortality from the group challenged with the reassortant virus was comparable to the group challenged with rNVI15 and considerably higher than the group challenged with Sp103. There are to date no published data to corroborate VP1 as a virulence determinant for IPNV.

Segment A encoded proteins

As discussed previously, genomic segment A encodes a 107 kDa polyprotein with a protein coding order of NH2–pVP2–VP4–VP3–COOH (Fig. 1.4). This polyprotein is co-translationally cleaved by the viral protease VP4 to produce the viral capsid proteins, pVP2 and VP3, releasing them in the infected cell. Petit *et al.* (2000) concluded that the protease cleavage site at the pVP2–VP4 junction (N-terminus) was located between amino acids 508 (alanine) and 509 (serine), and the

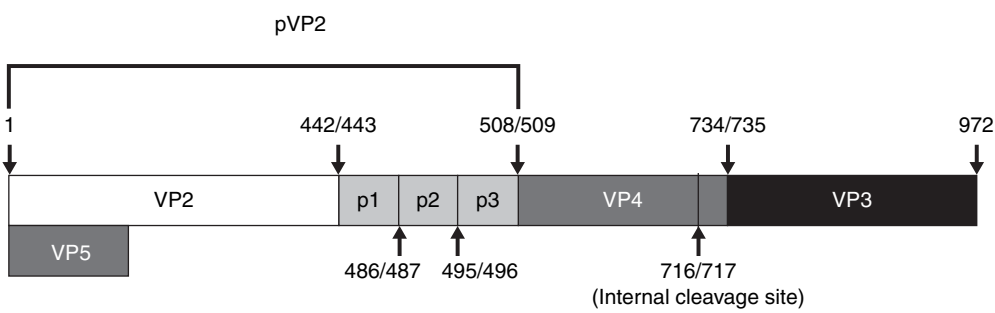


Fig. 1.4. The IPNV gene segment A polyprotein detailing the location of the major capsid protein, VP2, the three propeptides of VP2 (p1, p2, p3), VP4 viral protease, VP3 structural protein and the VP5 non-structural protein, which is positioned on a smaller overlapping ORF in a different reading frame. The cleavage sites are indicated by black arrows (figure modified from Lee *et al.*, 2007).

cleavage at the VP4–VP3 junction (C-terminus) occurred between amino acids 734 (alanine) and 735 (serine). It was discovered early in the molecular studies of IPNV that the pVP2 underwent further cleaveage near the carboxyl end to generate the mature VP2 protein (Dobos *et al.*, 1977). Subsequent studies revealed three cleavage sites within pVP2 located after amino acid residues 442, 486 and 495 (Galloux *et al.*, 2004). The products originating from these cleavage events were documented as VP2 (aa 1–442) and three propeptides, p1 (aa 443–486), p2 (aa 487–495) and p3 (aa 496–508), which remained associated with the virion (Galloux *et al.*, 2004). Segment A also encodes an arginine-rich, non-structural protein, labelled VP5, from a smaller overlapping ORF, which is in a different reading frame.

VP2 protein

IPNV is internalized into the cell by receptor-mediated endocytosis and the major outer capsid protein VP2 is thought to contain the cell attachment sites (Dobos, 1995a; Kuznar *et al.*, 1995; Granzow *et al.*, 1997). It has been reported that the VP2 protein contains two variable epitopes, termed H8 and B9, positioned between amino acids 204–330, a conserved neutralization epitope, F2, which is dependent on amino acids 153–203, as well as the 204–330 region, and another conserved neutralization epitope, AS-1 (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Frost *et al.*, 1995). Heppell *et al.* (1995a) identified two hydrophilic hypervariable sections within the central portion of the VP2 coding region. This central variable domain represented amino acid residues 183–335 and this finding was later confirmed in a molecular study by Blake *et al.* (2001). These hydrophilic areas are likely to be important antigenic regions, as they will be present on the surface of the virus particle.

Molecular markers of IPNV virulence have been identified within the VP2 coding region. Bruslind and Reno (2000) detected threonine (Thr) at amino acid residue 217 in the most virulent among three American Buhl isolates. Santi *et al.* (2004) conducted complete nucleotide sequence analysis of segment A (Sp serotype/nine strains), as well as complete sequence analysis of segment B (three strains). Very few nucleotide and deduced amino acid sequence variations were detected in the genes encoding VP1, VP3 and VP4 proteins; however, several amino acid variations were detected in the VP2 coding region. They reported that isolates that produced high mortalities and severe pathological changes possessed residues Thr and alanine (Ala) at amino acid positions 217 and 221 of the VP2 gene, respectively. Shivappa *et al.* (2004) also reported virulence associated with amino acids at positions 217 and 221 of three Norwegian serotype Sp strains. It is interesting to note that both the 217 and 221 amino acid residues are located within the hypervariable VP2 region identified by Heppell *et al.* (1995a). However, Bain *et al.* (2008) reported that eight Scottish IPNV clinical outbreak isolates had proline (Pro) at position 217 and Ala at position 221. Similar findings were also reported from Irish IPNV clinical outbreak isolates that contained Pro217/Thr221 and Pro217/Ala221 (Ruane *et al.*, 2009a). These findings would suggest that other genetic factors of the virus, and/or combinations of host species and environmental factors, may have an effect on IPNV virulence. By implementing a reverse genetics system, Song *et al.* (2005) later confirmed that amino acid residues, Thr217 and Ala221 in VP2, played a significant role in determining the virulence of Norwegian IPNV serotype Sp strains. They also reported that residue 221 might be involved with the cell culture adaptation of the virus and that viral strains with Thr221 induced a carrier state of infection compared with Ala221, which produced a highly virulent strain with high associated mortalities. Song *et al.* (2005) hypothesized that Thr221 could become predominant due to selective pressure on the dynamic viral quasispecies by the host immune response or due to different cell tropism in acute infection (exocrine pancreatic and liver) versus latent infection (macrophages).

Conflicting views have been published with regard to VP2 glycosylation, the importance of which is that glycosylation can have a bearing on cell and species tropism as well as epitope structures, the latter aspect of relevance to vaccine development. It has been reported that six potential N-glycosylation sites exist in the pVP2 of IPNV serotype Sp isolates (Håvarstein *et al.*, 1990). However, Perez *et al.* (1996) stated that the VP2 of IPNV were not glycosylated. In contrast, Hjalmarsson and Everitt (1999) found evidence that O-glycosylation of VP2 occurred in RTG-2 cells and Espinoza *et al.* (2000) that it occurred in the cytosol of CHSE-214 cells. Rimstad (2003) speculated that the amount of VP2 glycosylation was limited due to a lack of migration pattern.

The crystal structure of IBVD has been determined as a T=13*l* icosahedral capsid composed of 260 trimers of viral protein (VP2) by a combination of electroncryomicroscopy and X-ray crystallography (Coulibaly *et al.*, 2005). After feeding the atomic model of the VP2 into the electron density map of the virion, it was determined that the capsid was composed exclusively of VP2. The VP2 subunit is folded into three distinct domains: base (B), shell (S) and projection (P) (Coulibaly *et al.*, 2005). They detail that the amino acids that determine cell culture adaptation, virulence and the area of greatest antigenic variation are positioned in domain P, on the most exposed loops or 'spikes'. All three amino acids associated with virulence have their side chains pointing outward away from the contact points, important for the VP2 fold and subunits that stabilize the virion, which may allow these residues to attach directly to a receptor of a target cell. This manuscript gives essential clues into the tentative crystal structure of IPNV and the location on the VP2 trimer of amino acids involved with virulence and tissue tropism.

Using the technique described above, recombinant expression of mature IPNV VP2 (serotype Sp) in the absence of VP3 leads to the formation of an icosahedral subviral particle (SVP), whose structure has been described as consisting of 20 VP2 trimers, organized with T=1 icosahedral symmetry (Coulibaly *et al.*, 2010). They extracted a VP2 trimer from the IPNV SVP and compared its organization against that of IBDV. These authors further state that IPNV-VP2 contains the same 3-D fold structure as IBDV SVP; however, the loops in the P domain which form the trimeric spikes differ in morphology, the spikes in IPNV being convex compared with the concave spikes of IBDV, and display a different interaction mechanism at the surface of the viral particle. Of great importance was the fact that the amino acids that governed virulence and tissue tropism clustered in different locations on the P domains of IPNV and IBDV. The VP2 amino acids, 217 and 221, known to be involved in IPNV virulence, were located in loop P_{BC}, which was identified as being the outermost loop at the apex of the VP2 spike. Due to their location, these two residues do not play a role in either influencing the morphology of the VP2 fold or maintaining the stability of the virion, which suggests that they may be involved directly with the attachment of the virion to the target cell. A conserved, exposed groove located at the S–P interface, at the base of the spike, which demonstrated a large negative electrostatic potential, was also detected. It was previously reported that this conserved groove, incorporating amino acids 94, 176, 179–182, 377–379, as well as amino acids 131–134 and 229–231 from the neighbouring subunit in the trimer, was essential for IBDV binding to cells (Delgui *et al.*, 2009). Coulibaly *et al.* (2010) has summarized these findings by suggesting that the birnaviruses might interact with two separate receptors. The one at the apex of the P domain spike (P_{BC} in IPNV) is responsible for cell attachment and has an influence on virulence and tissue tropism, and the second receptor, positioned in the conserved groove, is responsible for internalization into the target cell.

Further work is required to understand better the virulence mechanisms associated with the VP2 protein and the interaction that the VP2 undergoes with the host immune response. Both aspects are ultimately of importance for further vaccine development and for understanding and preventing the IPNV carrier status of salmonid fish.

VP3 protein

The VP3 is a minor capsid protein and is thought to be positioned internally within the viral capsid. VP3 was found to be absent from empty capsids that lacked viral RNA and was referred to as an internal protein (Dobos, 1995a). However, MAbs directed against VP3 were shown to react with linear epitopes (Tarrab *et al.*, 1993), and Nicholson (1993) suggested that a proportion of VP3 might be present on the virion surface. Hjalmarsson and Everitt (1999) identified VP3 containing ribonucleoprotein complexes using electron microscopy and they proposed that the C-terminal end of the protein was associated intimately with viral RNA. By applying a yeast two-hybrid system in association with co-immunoprecipitation, VP3 was later shown to be a self-interacting protein which also interacted with VP1 and dsRNA in an independent manner (Pedersen *et al.*, 2007). The authors hypothesized that the VP3 binding to the genomic dsRNA might provide a mechanism for avoiding the host cell immune system and also speculated that the multiple interaction properties of VP3 might suggest an involvement in viral replication and virion morphogenesis. This hypothesis is strengthened by the work of Imajoh *et al.*, (2007), who studied the protease activity of a Japanese marine birnavirus strain (Y-6) isolated from yellowtail. Using electron microscopy, they discovered that pVP2 and VP3 were required for the production of virus-like particles in transfected CHSE-214 cell cultures and, when a further cleaved VP3 was co-expressed alongside pVP2, virus-like particles were not formed.

VP4 (NS) protein

As described earlier, it is the function of the IPNV VP4 protease to cleave the 972 residue polyprotein with a protein coding order of NH2–pVP2–VP4–VP3–COOH. As detailed in Fig. 1.4, the VP4 cleaves its own N-terminal after amino acid 508 and its C-terminal after amino acid 734 (Petit *et al.*, 2000). These VP4 proteases recognize the cleavage site consensus (Ser/Thr)-X-Ala ↓ (Ser/Ala)-Gly motif. The same authors discovered an internal cleavage site between residues Ala716 and Lys717, and also reported that the conserved catalytic residues, Ser^{633} and Lys^{674}, were essential for the processing of the polypeptide. It appears that the birnavirus proteases represent a distinct type of serine protease, utilizing a Ser/Lys catalytic dyad mechanism to process the polyprotein (Birghan *et al.*, 2000; Lejal *et al.*, 2000; Petit *et al.*, 2000). The crystal structure of the IPNV VP4 has been studied more recently (Lee *et al.*, 2007). Two crystal structures were reported, a hexagonal form ($VP4^{hex}$) that contained the free binding site of the VP4 protease and a triclinic crystal form ($VP4^{tri}$), whose active site revealed an acyl–enzyme complex formed with an internal VP4 cleavage site which allowed the identification of the substrate binding sites (S1, S3, S5 and S6).

VP5 protein

The smaller overlapping ORF on segment A encodes an arginine rich, minor nonstructural polypeptide, VP5 (15 kDa) (Magyar and Dobos, 1994). The VP5 has been detected in IPNV infected cells (Magyar and Dobos, 1994) but, to date, it has not been detected in purified virions. It has also been demonstrated by reverse genetics that this protein is not required for viral replication *in vitro* (Weber *et al.*, 2001) and several IPNV field isolates have been discovered which lack the initiation codon for VP5 (Heppell *et al.*, 1995b; Santi *et al.*, 2004). Sequence analysis by Santi *et al.* (2004) on IPNV serotype Sp strains found a high degree of variation within the 15 kDa ORF and that several strains contained an additional in-frame stop codon (UGA) at nucleotide 472, which coded a truncated 12 kDa VP5. These truncated VP5 isolates were found to be highly virulent; however, it was demonstrated that the VP5 protein was not required for viral replication *in vivo* and its

absence did not affect the virulence of the virus or the infection status of the Atlantic salmon fry (Santi *et al.*, 2005a).

Hong *et al.* (2002) documented that the VP5 had an anti-apoptotic activity in CHSE-214 cell cultures. It is well known that the Bcl-2 class of intracellular proteins plays a crucial role in the regulation of the apoptotic process (Gross *et al.*, 1999; Cory and Adams, 2002), and Hong *et al.* (2002) have discovered that the VP5 contains a Bcl-2 domain variant and hypothesize that this can enhance virus production. It was later reported that the VP5 of IBDV was able to suppress virus-induced apoptosis during the early stages of infection (Liu and Vakharia, 2006). Further experimental work is required to understand better the function of the VP5 protein of IPNV.

Replication

Many aspects relating to viral replication have been discussed in relation to the viral proteins and this section will describe briefly viral attachment, entry, uncoating of the virion, RNA synthesis and maturation.

An important aspect of the dsRNA viruses is that, throughout the replication cycle, their genome remains protected within the viral capsid and they themselves perform enzymatic activities such as transcription (Patton and Spencer, 2000). As a result, dsRNA virions must be translocated across the cell membrane without disassembling completely during entry into the host cell. Chevalier *et al.* (2002) determined that the maturation of birnavirus pVP2 into VP2 only took place at the time of viral assembly. The late maturation of VP2 into 25 nm particles may indicate that this is a deliberate act to avoid premature assembly during the formation of the virion and it could be speculated that the three associated peptides as described by Galloux *et al.* (2004) may assist with viral entry by destabilizing the cell membrane. It is also well understood that, due to the low proofreading efficiency of viral RNA polymerases, RNA viruses have the potential for high mutation rates during genome replication and are thought to exist as dynamic mutant variants termed quasispecies (Domingo and Holland, 1997).

Very early in the study of IPNV, Moss and Gravell (1969) conducted a comprehensive electron microscopy study of virion assembly and localization inside the cells. They found mature virions 6 h post-infection and at the time speculated that the causative agent might belong to the *Reoviridae* family.

The majority of the work studying IPNV replication has been performed using the CHSE-214 and BF-2 teleost cell lines. It has been demonstrated that IPNV replication occurs in the cytoplasm of cells, and a single replication cycle in CHSE-214 cells takes about 16–20 h, depending on incubation temperature (Somogyi and Dobos, 1980). IPN virions attach both specifically and non-specifically to CHSE-214 cell membrane components and competition experiments using inactivated virions demonstrate that specific binding is required for productive infection (Kuznar *et al.*, 1995). The same authors found that at 20°C, adsorption and entry into the cells were accomplished within 20 min. IPNV is then internalized into the vesicular compartments of the cytoplasm (Couve *et al.*, 1992) by receptor-mediated endocytosis (Granzow *et al.*, 1997). As detailed previously, the IPNV RdRp was active without proteolytic pretreatment of the virus, indicating that uncoating may not be required for viral replication (Cohen, 1975). A 14–16S transcription intermediate is formed 2–4 h after infection and viral RNA can be detected 4–6 h post-infection (Somogyi and Dobos, 1980). Viral protein synthesis in comparable proportions also occurred during this period. The amount of synthesized viral RNA reached peak levels 8–10 h post-infection, which coincided with the detection of genomic dsRNA. The synthesis of viral RNA decreased 14–16 h post-infection (Dobos, 1977).

A recent study into the molecular characterization of replication intermediates (RI) from IPNV isolates inoculated on to CHSE-214 cells identified two viral ribonucleoprotein (RNP) complexes: one with a CsCl buoyant density of 1.33 g/cm^3, most probably

nucleic acids of mature viral particles, and a second viral RNP complex with a higher molecular weight (CsCl buoyant density > 1.4 g/cm^3), present in infected cells 4 h post-infection (Cortez-San Martín *et al.*, 2009). Radiolabelling with [^{32}P]HPO_4^{2-} revealed a higher amount of viral RNAs within this higher molecular weight viral RNP complex than associated with viral particles and that peak levels were reached 18 h post-infection. Tris-glycine agarose electrophoresis of this viral RNP complex produced a smeared lane, indicating replicating viral RNAs, and SDS-Page analysis after the fractions had undergone RNase V1 treatment resulted in a single radioactive band of approximately 94 kDa, consistent with the molecular weight of IPNV VP1. Further analysis revealed the presence of dsRNA 7 h post-infection and detected both dsRNA and ssRNA from samples collected 24 h post-infection. From these results, it would appear that two viral RNP complexes existed in infected cells, one which contained mature viral particles, complete with dsRNA, and a second containing the RdRp (VP1/VPg) and a mixed population of single- and double-stranded viral RNA species, most probably indicative of RNA RIs involved in viral RNA synthesis. Northern blot analysis of total RNA indicated viral RNA RIs which utilized the full-length negative-strand as the template for the synthesis of various intermediate length positive-strands of viral RNA where there was incomplete synthesis at the 3′-terminal.

Espinoza and Kuznar (2010) highlighted that IPN viral RNAs could be stained using the TUNEL kit, as fluorescence was observed during the virus life cycle in the cytoplasm of IPNV infected CHSE-214 cells. Infected cells were also double-labelled with an IPNV VP3 MAb and the terminal deoxynucleotidyl transferase (TdT) enzyme used in the TUNEL assay. They discovered that the majority of cells that stained MAb VP3 protein positive were also labelled by TUNEL fluorescence in the cytoplasm of the cells. In addition, in a small number of cells, both labels co-localized in one or two bigger foci, presenting an intense fluorescence. As the VP3 protein was required for virion packaging, they speculated that these double-labelled foci could be viral assembly sites or factories within the cell. Therefore, the TUNEL assay potentially could enable the visualization of viral RNA containing intermediates during IPNV replication.

Villanueva *et al.* (2004) identified two types of viral particles (termed A and B) during the assembly process of the virions. They hypothesized that the genomic ds-RNA was assembled into larger (66 nm diameter) non-infectious particles (particles A), whose capsid contained both mature and immature polypeptides. They reported that these provirions were composed of the viral genome but that viral assembly was incomplete, indicating that they corresponded to immature intermediates of morphogenesis. These immature provirions then underwent a proteolytic cleavage of most of the remaining viral precursors in the capsid to form infectious particles B (complete virions) 3–4 h later during the viral cycle. Thirty years earlier, Nicholson and Dunn (1974) demonstrated that viral replication *in vitro* was reduced due to the presence of defective interfering (DI) particles. Later work revealed that these DI particles appeared when cell cultures were infected at higher titres (MacDonald and Kennedy, 1979) and that they had the ability to create persistently infected cell cultures unable to produce CPE (Hedrick and Fryer, 1981). It is tempting to speculate that these so-called DI particles and the immature particle A reported by Villanueva *et al.* (2004) are identical.

The mechanisms involved with IPNV replication, genome and particle assembly must be understood in greater detail to assist zoo-sanitary and health management regimes, as well as vaccine development (see later sections). Reverse genetics systems should be further utilized to increase our knowledge of the role(s) of each protein and the interaction between virus and host.

Diagnostic Methods

Historically, the diagnosis of IPN (both the clinical disease and the carrier state) was

based on isolation of the virus on cell culture, followed by antibody-based identification of the agent. Over the years, numerous immunological techniques have been developed and improved to facilitate quantitative outcome reading and the demonstration of IPNV *in situ*. More recently, rapid and sensitive molecular methods have gained popularity, equally including techniques that yield quantitative outcomes, and are applicable *in situ* (Table 1.6).

The methods (Table 1.6) have varying specificity and sensitivity features and the success of any given method will vary depending on the viral titre present in the tissues or cells to be examined and optimization of sample selection and processing to fit the specific diagnostic purpose.

The majority of IPNV diagnostic testing methods require sampling of organs that cannot be obtained without sacrificing the donor animal, although some methodologies using non-destructive samples (blood, reproductive fluids) have been documented. It is fair to say that cell culture assays have proved to be a very sensitive and consistent method for isolating aquatic birnaviruses and that a large number of fish disease laboratories use cell culture isolation as part of their diagnostic routines. It is also the only diagnostic principle yielding firm proof that the detected agent is alive and infectious. Nevertheless, it is still a relatively expensive and laborious method which takes 14 days or more to produce a definitive outcome. With the increased diversity of methods available, the specific needs of the diagnostic situation today can often be served as well or better using faster and less costly methods.

Tissue culture isolation

A comprehensive summary of the vast amount of scientific literature on cell culture isolation of IPNV has been given by Reno (1999) and will therefore not be repeated here. In general, there has been no information to question significantly the conclusions made therein, and the details of the cell culture method as described by the OIE (Anon., 2006) and the American Fisheries

Table 1.6. Principal methods documented for diagnosis of IPN.

Diagnostic principle	Diagnostic technique	References
Viral isolation	Cell culture propagation	Anonymous (2006)
Antibody binding to virion epitopes	Neutralization assay	Lientz and Springer (1973)
	FAT/IFAT	Tu *et al.* (1974); Swanson and Gillespie (1981)
	ELISA	Nicholson and Caswell (1982); Dixon and Hill (1983a)
	Immunohistochemistry (IHC)	Ahne (1981); Evensen and Rimstad (1990)
	Immunodot blot	McAllister and Schill (1986)
	Flow cytometry	Rodriguez Saint-Jean *et al.* (1991)
	Coagglutinaton test	Kimura *et al.* (1984); Taksdal and Thorud (1999)
Amplification and detection of viral nucleic acids	RT-PCR	Lopez-Lastra *et al.* (1994); Blake *et al.* (1995); Wang *et al.* (1997); Taksdal *et al.* (2001)
	In situ hybridization	Biering and Bergh (1996); McCarthy *et al.* (2008)
	Real-time RT-PCR	McBeath *et al.* (2007); Bowers *et al.* (2008)
	RT-LAMP	Soliman *et al.* (2009)
Detection of fish antibody response	Neutralization assays	Wolf *et al.* (1963); Yamamoto (1975a)
	ELISA	Dixon and de Groot (1996)

Society (Anon., 2007) still represent the 'gold standard' for IPN diagnosis in salmonid fish. Aquatic birnaviruses are grown easily in susceptible cell lines at 10–25°C and produce quite consistently a characteristic cytopathic effect (CPE) within a few days, which greatly simplifies the outcome reading. Numerous cell lines are permissible for IPNV (reviewed by Reno, 1999) and, in general, cell culture is among the most sensitive techniques to detect low IPNV concentrations, clearly more sensitive than most, if not all, antibody-binding assays.

A comparison of the relative susceptibility of five teleost cell lines to IPNV was made based on data from an interlaboratory proficiency exercise involving 11 European National Reference Laboratories (Lorenzen *et al.*, 1999). The authors found that the CHSE-214 cell line was the most sensitive to IPNV infection, followed by BF-2 and RTG-2 (Table 1.7). However, there was a considerable variability in the diagnostic sensitivity between the participants. McAllister (1997) found that the susceptibility of cell culture would also vary between different laboratories and attributed this in part to differing cell lineages and/or various factors concerned with the culture of the cells, e.g. incubation temperature, incubation time, culture media, addition of sera/buffers etc. Interestingly, Fourrier *et al.* (2007) reported that CHSE-214 cells infected with IPNV serotype Sp demonstrated a significant loss in their ability to produce CPE around passage 290 and laboratories were therefore advised to monitor the sensitivity of their diagnostic cell lines routinely.

Table 1.7. An overview of the 'standard' cell lines used in the isolation of IPNV and their susceptibility (after Lorenzen *et al.*, 1999). Results obtained from 11 European National Reference Laboratories during an interlaboratory comparison of the susceptibility of five selected cell lines to IPNV.

Cell line	Species of origin	IPNV titration median LOD (log_{10} $TCID_{50}$/ml)
BF-2	Bluegill (*Lepomis marcrochirus*)	5.24
CHSE-214	Chinook salmon (*Oncorhynchus tshawytscha*)	5.40
EPC	Common carp (*Cyprinus carpio*)	3.32
FHM	Fathead minnow (*Pimephales promelas*)	2.90

The standard processing procedure for aquatic birnavirus isolation is tissue homogenization, followed by low speed centrifugation before inoculation of the two dilutions of the supernatant on to the cell culture, one passage after 7 days and readout after another 7 days. Tissue processing has been optimized, including: homogenization techniques (McAllister *et al.*, 1993), sonication of the cell pellet (McAllister *et al.*, 1987; Smail *et al.*, 2003) and co-cultivation using trypsinized tissue material (Agius *et al.*, 1982). The concentration of macrophages and treatment to release their contents has brought improvements for the diagnosis of asymptomatic carrier fish. Munro *et al.* (2004) used lysates of either kidney macrophages or blood leucocytes to optimize sensitivity, and Gahlawat *et al.* (2004) found that lysates of isolated blood leucocytes were superior in detecting carrier Atlantic halibut compared with standard culture or RT-PCR from kidney homogenates. Johansson and Olesen (2009) confirmed the benefits of the macrophage lysis method. Viral titres in tissue material of diseased fry can be as high as 10^8–10^9 $TCID_{50}$/g and range down to the limit of detection of the cell culture assay for asymptomatic carrier fish (50–100 $TCID_{50}$/g) (Smail and Munro, 2001).

As mentioned previously, IPNV titres will decrease when subjected to a freeze/thaw process. It is therefore important not to freeze tissues when submitting samples and to use 50% glycerol to preserve the sample during transit, as recommended by Wolf and Quimby (1971).

As the CPE induced by IPNV is a non-specific result, most laboratories use a second confirmatory technique to ascertain the CPE was caused by IPNV. While neutralizaton tests were initially dominant, rapid antibody-based (Garden *et al.*, 2008) or nucleic

acid detection methods to confirm that cultures were indeed IPNV infected were developed. Routine confirmatory testing independent of CPE, or prolonged incubation of second passage cultures can be employed to improve the detection of slowly replicating or non-CPE-inducing isolates.

Antibody-binding assays

Neutralization assays

The ability of antibodies raised in mammalian species to neutralize the cell culture infectivity of IPNV was first reported by Lientz and Springer (1973) and this method is still the most sensitive among the antibody-based assays for IPNV. Due to its sensitivity, it has found wide use in virus characterization and typing using polyclonal or monoclonal antibodies (Caswell-Reno *et al.*, 1986; Christie *et al.*, 1990; Hill and Way, 1995). The main disadvantage is time and costs due to the use of cell culture for readout. Neutralization assays were also among the first to demonstrate circulating antibodies against IPNV in salmonids (Wolf *et al.*, 1963; Yamamoto, 1975a).

ELISA

This method allows for the important benefits of rapid processing (1.5–3 h) and a high throughput of samples. Dixon and Hill (1983a) described a sandwich ELISA using an unlabelled rabbit polyclonal antibody for capture and another (labelled) for binding, and other groups have followed (Rodák *et al.*, 1988; Davis *et al.*, 1994; Smail *et al.*, 2003). Several IPNV ELISA kits are commercially available with estimated limits of detection (LOD) from 10^3 pfu/ml (virus culture) to 10^5 pfu/g (tissues). Monoclonal antibodies can be used to improve the specificity of ELISA assays; however, it is worth remembering that individual MAbs may not cross-react with all strains within serogroup A. An ELISA for detection of fish antibodies against IPNV has been described by Dixon and de Groot (1996).

Immunofluorescent assays (FAT/IFAT)

Tu *et al.* (1974) developed an IFAT assay using FITC-labelled rabbit polyclonal antisera for the confirmation and quantification of IPNV in infected RTG-2 cell cultures, and Swanson and Gillespie (1981) documented the method for virus in tissues. Indirect immunofluorescence antibody assay (IFAT) utilizing monoclonal antibodies was used to characterize Scottish IPNV isolates (Smail *et al.*, 2006). Osorio *et al.* (2005) went on to demonstrate that fluorescent microscopy also could be used to label and enumerate IPNV particles in cell-free suspensions and suggested that this method be used to study the early interaction between IPN virions and their host cells. IFAT assays are used routinely to detect a variety of viral pathogens and are among the generic methods used in fish health laboratories. However, experienced laboratory analysts are required to read the stained samples, as this assay can produce a certain amount of non-specific background staining. It requires fresh or frozen samples and cannot be performed on formalin-fixed material, which represents a limitation to its use.

Immunohistochemistry (IHC)

Immunostaining using horseradish peroxidase- or alkaline phosphatase-labelled antibodies is a widely used method to confirm IPNV infection in tissues. The major advantage of this method is that the virus can be visualized within the pathological changes (Evensen and Rimstad, 1990) and thereby establish IPNV plausibly as the cause of disease. However, there is the potential for background (artefact) staining due to the presence of endogenous enzymes in the tissue sections. IHC can be performed in less than 24 h and can also be used on fixed tissue sections. Its sensitivity is generally similar to IFAT (Ahne, 1981; Rodriguez Saint-Jean *et al.*, 2001).

Coagglutination test

Using formalin-fixed *Staphylococcus aureus* cells sensitized with a polyclonal rabbit

anti-IPNV serum, Kimura *et al.* (1984) reported the use of a coagglutination test producing aggregation of the bacteria (visible clumping) in the presence of viral antigen. The method was validated for use in clinical diagnosis by Taksdal and Thorud (1999) and for confirming the presence of IPNV in cell culture supernatants (Garden *et al.*, 2008), in both cases having an estimated LOD of approximately 10^5 $TCID_{50}$/g. Its major advantage is that it does not require any specific equipment and gives an immediate result. Moreover, similar coagglutination assays can also be prepared using beads instead of *S. aureus* to carry the reactive antibody.

Immunodot blot

Adsorption of sample suspension on to nitrocellulose strips followed by incubation with anti-IPNV (primary) antibodies and subsequent detection using enzyme-conjugated (secondary) antibodies was shown to detect IPNV derived from cell cultures and from tissues (Hsu *et al.*, 1989). Kirsinger *et al.* (1999) employed this method with monoclonal antibodies and found its sensitivity comparable to ELISA, the main advantage being the ability to evaluate inoculated cell cultures within 14 h post-inoculation, before the onset of CPE.

Flow cytometry

Spanish workers have published on the use of the flow cytometry technique to detect IPNV from cell suspensions first incubated with anti-IPNV rabbit antiserum and then with an FITC-labelled conjugate. The method has proven suitable for use with purified rainbow trout leucocytes (Rodriguez Saint-Jean *et al.*, 1991) and spermatozoa samples (Rodriguez Saint-Jean *et al.*, 1993). However, this technique obviously requires a relatively expensive flow cytometer and highly skilled personnel to operate the equipment and interpret the results.

A study comparing the 'benchtop' sensitivity of five antibody-based assays (IFAT, flow cytometry, immunoperoxidase assay, immunodot blot and coagglutination test) and an RT-PCR assay for IPNV detection has been undertaken by Rodriguez Saint-Jean *et al.* (2001). They reported that the RT-PCR method was the most sensitive in detecting IPNV infected cell cultures with an LOD of 1×10^1 $TCID_{50}$/ml of culture supernatant, followed by flow cytometry and IFAT (both LOD 1×10^2 $TCID_{50}$/ml).

Molecular methods

Since the mid-1990s, the development and use of molecular methods for detecting IPNV nucleic acids have become increasingly popular (Table 1.6), most of which use primers developed against parts of the VP2 gene or targeting the VP4–VP3 encoding junction.

Reverse transcription-polymerase chain reaction (RT-PCR)

Early studies to calculate the sensitivity of IPNV RT-PCR methods mainly used serial dilutions of extracted RNA to calculate the LOD (Lopez-Lastra *et al.*, 1994; Suzuki *et al.*, 1997; Wang *et al.*, 1997), while others compared their RT-PCR assay against traditional cell culture isolation (Blake *et al.*, 1995; Taksdal *et al.*, 2001). It is important to remember that the LOD achieved by using purified viral RNA may not represent the actual LOD when screening tissue material, as tissue samples undoubtedly will contain inhibitory substances and the samples may also be compromised during transportation. A study to optimize and validate a conventional IPNV RT-PCR was conducted by Kerr and Cunningham (2006) using the primer set described by Taksdal *et al.* (2001). Using optimized procedures, the assay correctly identified IPNV isolates from serotypes A1–A9 and detected as few as 10 infectious units/g kidney.

While conventional RT-PCR relies on gel electorphoresis and staining to confirm amplification, RT-PCR can also be carried out with ELISA readouts (Milne *et al.*, 2006). This technique requires biotinylation of one IPNV-specific PCR primer by

the use of digoxigenin-dUTP during PCR amplification. The resulting dig-dUTP labelled product can then be captured in streptavidin pre-coated wells and analysed by standard ELISA colorimetry. The authors reported that this RT-PCR ELISA method could successfully detect 1.5×10^4 pfu/ml IPNV in spiked kidney tissue. Also, Barlič-Maganja *et al.* (2002) reported the successful use of an RT-PCR ELISA technique based on Blake *et al.* (1995) primers, claiming its sensitivity would be comparable to cell culture.

Multiplex RT-PCR assays

This assay type has the advantage that it screens for several pathogens simultaneously and scientists have documented its use in detecting IPNV (Yoshinaka *et al.*, 1998). Williams *et al.* (1999) reported the development of a multiplex RT-PCR assay for the detection of IPNV, infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia (VHSV), with a sensitivity of 100 $TCID_{50}$/ml, from IPNV tissue culture supernatant. The major advantage of this system compared with RT-PCR is that reasonable savings in time, sample material and cost can be made.

Real-time (RT-)PCR and RT-LAMP assays

In the past few years, several groups have published on IPNV real-time RT-PCR assays, which include optical readout systems that are being read (quantitatively) as the amplification reaction processes. Bowers *et al.* (2008) described a real-time PCR assay using the SYBR Green I fluorescent dye that was used to detect and quantify IPNV in spleen, head kidney and pectoral fin tissues of IPNV-challenged rainbow trout. They used primers designed against the VP4 gene and were able to detect *in vitro* transcribed RNA down to 10 RNA copies (0.01 fg). The assay was capable of detecting IPNV in challenged fish that did not show any clinical signs of infection, suggesting that it could be used to detect asymptomatic carriers. A TaqMan® probe real-time PCR assay for the detection of IPNV was documented on experimentally challenged Atlantic salmon post-smolts (McBeath *et al.*, 2007). They used primers and a probe designed against IPNV Sp VP2 and were able to detect fish exposed by intraperitoneal (i.p.) injection, as well as by cohabitation. The method's main advantage over conventional RT-PCR is its automation potential, improved speed and its quantitative readout, but it requires significant investment in laboratory equipment. For good quantification, real-time PCR assays require normalization of outcome readings relative to values obtained with tissue reference ('housekeeping') genes. A recent study by Julin *et al.* (2009) showed that the stability of candidate reference genes varied over the time course of IPNV infection in tissues and cell cultures, suggesting that tissue-specific combinations of housekeeping genes should be used for normalization rather than one 'universal' reference gene.

Soliman *et al.* (2009) have recently developed a sensitive and rapid one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for IPNV detection. This assay uses six IPNV-specific primers designed against the VP4/VP3 junction area to amplify target nucleic acid under isothermal conditions (65°C). The six primers can recognize up to eight distinct areas on the target genome and the primers initiate a self-recurring strand-displacement DNA synthesis of the target material. The LAMP products can be read visually within 1 h using either SYBR Green I stain or a fluorescent detection reagent. The authors reported that this assay had a considerably higher analytical sensitivity than RT-PCR when detecting purified viral RNA and that the LOD was a supernatant dilution of 10^{12}, which would equate to approximately 0.001 fg RNA. As compared to the real-time RT-PCR, its key features are simplicity, speed and no need for expensive laboratory equipment, making it feasible for use with local fish health services or in quality assurance laboratories of fish farming companies.

In situ *hybridization*

In situ hybridization techniques can be used alongside or instead of standard IHC staining.

This method differs from IHC as it uses a molecular probe to attach to genomic material of the target pathogen on tissue sections or cell cultures. Colorimetric *in situ* hybridization methods have been documented for the diagnosis of IPNV (Biering and Bergh, 1996). More recently, McCarthy *et al*. (2008) described a fluorescent *in situ* hybridization (FISH) method for IPNV detection. As is the case with IHC, background staining or autofluorescence (FISH technique) can pose a problem to diagnosticians. The benefits described in the IHC section also apply to the ISH/FISH technique.

Antibody detection

Compared with terrestrial animals, the use of antibody detection assays for diagnostic purposes is uncommon in fish, although both neutralization assays (Lientz and Springer, 1973) and ELISA techniques (Dixon and de Groot, 1996) for anti-IPNV antibodies are well documented. In our opinion, this is due at least in part to the lack of reliable correlation between the absence or presence of antibody and the absence or presence of virus carriage (see later in this chapter for a discussion). Another important factor is the seasonal variability inherent with the temperature-dependence of antibody levels and responses in poikilothermic animals.

Summary of diagnostic methods

In summary, it must be emphasized that the choice of method(s) for IPN diagnosis must be made according to the intended use of the assay outcome (Table 1.8). If the purpose is to confirm or reject IPN as a clinical diagnosis, confirmation should be sought using histopathology followed by IHC or ISH/FISH to demonstrate abundant virus associated with pathological lesions. Where a farm is undergoing an IPN outbreak, there are high viral titres in target organs and the diagnostic method applied will not require a high degree of sensitivity. Indeed, ELISA or the *S. aureus* coagglutination test may be the method of choice as they allow for rapid detection and often yield negative outcomes if titres are below those commonly found in clinically diseased animals. Wherever IPNV carriers are abundant, positive cell culture or PCR tests may lead to erroneous IPN diagnosis if not supported by titre assessment. The IPNV coagglutination test has the added advantage that it can be used in the field. Direct application of a droplet of ascites fluid from diseased or moribund fish frequently yields positive results, even

Table 1.8. Rapid diagnostic methods for detecting IPNV, *in vitro* sensitivity and their suitability for screening diseased and/or asymptomatic carriers.

Diagnostic test	Detection limits (*in vitro*)	Detects IPNV in diseased fish?	Detects IPNV in carrier fish?	Reference
ELISA	1.5×10^3–1.5×10^4 $TCID_{50}$/ml	Yes	No	Dixon and Hill (1983a); Rodák *et al*. (1988)
Flow cytometry	$1\times10^2TCID_{50}$/ml	Yes	Yes	Rodriguez Saint-Jean *et al*. (2001)
IFAT	$1\times10^2TCID_{50}$/ml	Yes	Yes	Rodriguez Saint-Jean *et al*. (2001)
RT-PCR	15 fg–1 pg (of purified viral RNA)	Yes	Yes	Lopez-Lastra *et al*. (1994); Wang *et al*. (1997)
Nested-PCR	1 fg	Yes	Yes	Suzuki *et al*. (1997)
Real-time PCR	0.01 fg	Yes	Yes	Bowers *et al*. (2008)
RT-LAMP	0.001 fg	Yes	Yes	Soliman *et al*. (2009)

without tissue homogenization, as seen when testing fish dying after experimental induction of the disease (Anne Ramstad, personal communication).

On the other hand, if the aim of the diagnostic investigation is to detect individual asymptomatic IPN-carrier fish or to screen valuable broodstock, then maximal sensitivity is called for, using the optimal tissues (blood leucocytes and/or kidney macrophages) and cell culture, or the most sensitive methods for nucleic acid detection such as nested PCR, real-time RT-PCR or RT-LAMP.

Pathogenesis, Immune Mechanisms and Induction of Disease

Port of entry

The attachment and entry of IPNV into fish tissues has been studied mainly using cell culture models (see Rodriguez Saint-Jean *et al.*, 2003), and the evidence for specific receptor-mediated uptake is accumulating (Ørpetveit *et al.*, 2008). Martin-Armas *et al.* (2007) studied the uptake and intracellular processing of aquatic birnaviruses by Atlantic cod scavenger endothelial cells (SEC) *ex vivo*, but found that none of the major endocytosis receptors was involved. The role of specific receptor binding for IPNV infection *in vivo* therefore remains to be proven. The fact that very low concentrations of IPNV are needed for successful challenge via water suggests that very efficient mechanisms for active uptake of the virus are present in gill, intestinal mucus and/or cutaneous tissues.

Tissue tropism and tissue damage

Early publications on IPN described the intestinal (McGonigle, 1940) and pancreatic (Wood *et al.*, 1955) lesions that gave this disease its name, and Sano (1971) documented tissue damage in excretory and haematopoietic renal tissue during acute IPN infection. Swanson and Gillespie (1981) developed an indirect fluorescent antibody procedure to detect IPNV in tissue sections and this technique, along with virus isolation and conventional histopathology, was used to study the tissue tropism of IPNV following experimental infection in brook trout, rainbow trout and Atlantic salmon (Swanson, 1981). Within 2–3 days after i.p. challenge, IPNV was demonstrated in kidney and intestinal tissues, where it replicated to high titres and strong fluorescent signals developed, especially in pancreatic acinar tissue (Swanson and Gillespie, 1979; Swanson *et al.*, 1982). The authors also noted fluorescence in kidney cells, along with the presence of debris-laden macrophages, and hypothesized that the virus was transported there by phagocytic cells. They continued to demonstrate the presence of IPNV in mononuclear peripheral blood cells, thereby documenting the role of viraemia in the pathogenesis of IPN (Swanson and Gillespie, 1982). During acute IPN, viral replication in pancreatic, intestinal and kidney tissues can yield titres $>10^{10}$ $TCID_{50}$/ml (Smail *et al.*, 1995, 2006), coinciding with considerable cell necrosis. While kidney titres up to 10^5 $TCID_{50}$/ml are not uncommon in apparently healthy fish, most salmonids succumbing to clinical IPN will have higher titres in the target tissues (Wolf, 1988; Taksdal and Thorud, 1999).

The severe necrosis of the intestinal mucosa (Fig. 1.5) and exocrine pancreas (Fig. 1.6) (McKnight and Roberts, 1976; Roberts and McKnight, 1976; Smail *et al.*, 1992) is believed to cause anorexia, as well as chronic maladaptation ('pinheads' or 'failing smolts'); a phenomenon often seen in a proportion of the survivors after an IPN outbreak (Smail *et al.*, 1995). The involvement of hepatic tissue damage (Fig. 1.7) in clinical IPN was noted by Swanson (1981) and described in greater detail by Taksdal *et al.* (1997). Roberts and Pearson (2005) reviewed the histology of 21 IPN outbreaks from Scottish farmed Atlantic salmon post-smolts and reported liver lesions as severe focal or generalized necrosis and loss of characteristic cellular architecture and inflammatory infiltrates, although no conclusive evidence of association to the virus was presented.

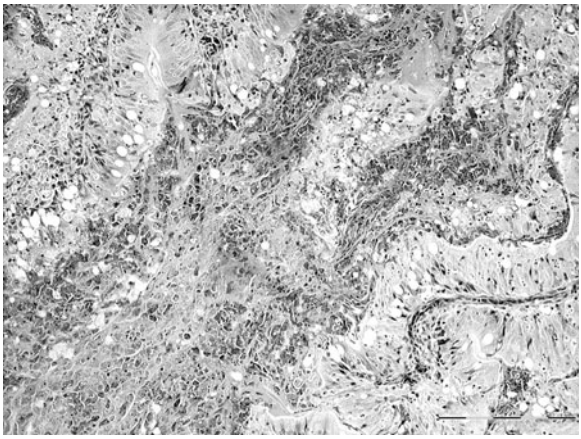


Fig. 1.5. Acute catarrhal enteritis of the intestinal mucosa. Bar scale=20 µm. Image supplied by Patricia Noguera (original), Marine Scotland, Scotland.

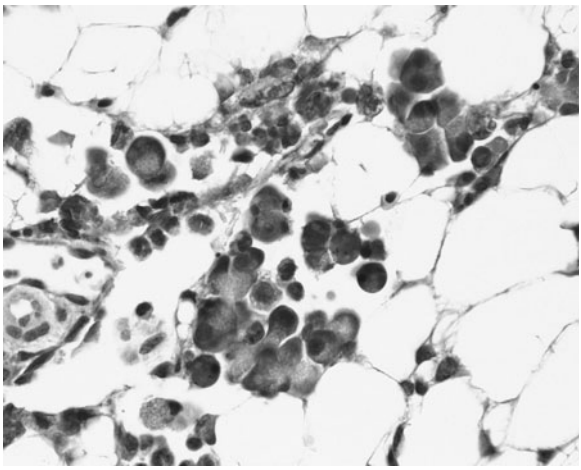


Fig. 1.6. Necrotic lesions in Atlantic salmon pancreas with bright red IHC staining indicating the presence of IPNV antigen within the damaged tissue. Image supplied by Prof Øystein Evensen (original), The Norwegian School of Veterinary Science, Norway.

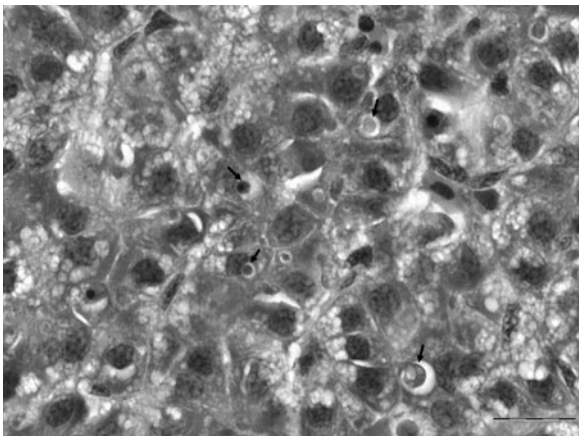


Fig. 1.7. Generalized hepatocyte degeneration, fine vacuolation and apoptotic bodies (arrowed). Bar scale=20 µm. Image supplied by Patricia Noguera (original), Marine Scotland, Scotland.

They emphasized an extensive acute necrosis of hepatocytes as a new and distinctive syndrome of Scottish Atlantic salmon post-smolts suffering from IPN. A further description of the liver involvement in Atlantic salmon post-smolts and analysis of the role in the pathogenesis of IPN were presented by Noguera (2006). The liver therefore contributes to the nature of infection and is an important factor in the disease process and fish survival. Smail *et al.* (2006) confirmed this finding and showed that IPNV antigen could be demonstrated in necrotic liver tissue of diseased smolts by immunohistochemistry, suggesting extensive virus replication in this cell type. It is obvious that cell necrosis of the digestive glands and of mucosal gut epithelium is responsible for shedding of infective virus particles into the environment with faeces. According to the view of McKnight and Roberts (1976), intestinal damage plays a dominant part in IPN-related mortality.

Recently, the ability of IPNV to induce programmed cell death *in vivo* has been shown by several groups (Eléouët *et al.*, 2001; Imatoh *et al.*, 2005; Santi *et al.*, 2005b) demonstrating apoptosis markers in hepatic, intestinal and pancreatic acinar tissues that simultaneously accumulate virus antigen and show typical pathological features. While Hong *et al.* (1998) postulated that apoptosis would precede cell necrosis based on cell culture studies, other workers found that the vast majority of cells derived from *in vivo* affected tissues displayed either the necrosis or the apoptosis marker (Imatoh *et al.*, 2005; Santi *et al.*, 2005b). Santi *et al.* (2005a) therefore hypothesized that apoptosis might play a role in limiting rather than aggravating the consequences of IPNV infection.

Roberts and McKnight (1976) noted extensive focal vacuolization in brain sections from rainbow trout with acute IPN, which would explain the erratic swimming often seen in diseased fry and fingerlings. Exophthalmos and ascites frequently seen in diseased fry suggest an underlying vascular damage, which also would be coherent with petechial haemorrhages observed in diseased fry (Wood *et al.*, 1955). There are no published investigations into this aspect of IPN pathogenesis, to confirm either the presence or possible replication of IPNV in mucus cells of the gills, as previously noted by Smail *et al.* (2006).

Immune responses

The immune responses mounted upon IPNV infection are readily measurable but nevertheless insufficient to provide effective protection. The expression used by Reno (1999), 'too little, too late', may serve to summarize immunity briefly, not only in fry but also in older salmonids. Sadasiv (1995) reviewed the immunological responses of salmonids to IPNV and emphasized the lack of viral clearance, despite the presence of neutralizing humoral antibodies, pointing towards the ability of the virus to infect leucocytes persistently as an explanation, and addressed a potential parallel to infectious bursal disease (IBD) of chicken. The recurrence of viral replication and clinical disease following stress (Roberts and McKnight, 1976; Stangeland *et al.*, 1996) and IPNV carriers being susceptible to superinfection (Taksdal *et al.*, 1998) are further examples that naturally aquired immunity against IPNV remains low.

Antibody responses that neutralize IPNV infectivity *in vitro* are demonstrated readily in naturally infected salmonids (Vestergård-Jørgensen, 1973a; Yamamoto, 1975a; Smail and Munro, 1985) and, although there is significant variability, neutralizing titres above 1000 have been reported (Mangunwiryo and Agius, 1988). Immunity mechanisms in poikilothermic animals are highly dependent on environmental temperature, but good neutralizing responses against IPNV have been found in rainbow trout maintained at 6°C (Sadasiv *et al.*, 1993). While the main neutralizing epitopes are located on the VP2 (capsid) protein (Tarrab *et al.*, 1995), Park and Jeong (1996) have found that VP3 also contained neutralizing sites. Frost *et al.* (1998) reported that both VP2 and VP3 epitopes were recognized readily by sera from IPNV-challenged Atlantic salmon with ELISA titres as high as $3.8 \times \log_{10}$. However, experimental evidence that anti-IPNV antibodies can neutralize

virus infectivity *in vivo* has not, to our knowledge, been published, except in a master's thesis (Agniel, 1975).

Knott and Munro (1986) noted that head kidney leucocytes (HKLs) of Atlantic salmon persistently infected with IPNV exhibited a poorer proliferative response to *in vitro* stimulation than did leucocytes from control fish, and this was confirmed in rainbow trout by Tate *et al.* (1990), who also noted that the mitogenic responses were stronger in the nylon wool-adherent, surface immunoglobulin-positive (Ig+) fraction than in the non-adherent Ig– fraction. Johansen and Sommer (1995) observed that the plastic adherent fraction of IPNV-infected salmon HKLs exhibited a somewhat reduced respiratory burst activity. In rainbow trout, Siwicki *et al.* (1998) demonstrated a moderate reduction of phagocytic and *A. salmonicida* killing capacity of blood leucocytes, along with reduced myeloperoxidase activity of these cells in IPNV infected fish and a slightly reduced respiratory burst activity of HKLs. However, feeding a dimerized lysozyme preparation over 2 weeks stimulated the immune cell activity to values above those of the control (non-infected) fish. In Atlantic salmon subjected to experimental infection, Rønneseth *et al.* (2006) showed that among HKLs, the proportion of cells labelled with a neutrophilic cell marker declined, while the proportion labelled with a B-cell marker MAb was stable. IPNV infection did not appear to affect HKL phagocytic activity or complement activity in plasma of rainbow trout (Byrne *et al.*, 2008).

Interferons provide non-specific immunoprotection by inducing Mx protein expression in various cell types of fish (Arnheiter and Meier, 1990). Lockhart *et al.* (2004) demonstrated that IPNV infection induced a rapid and persistent expression of Mx-encoding genes in Atlantic salmon post-smolts, while similar expression was not detected in persistently infected growers. It was demonstrated that Mx expression could be induced as effectively in these growers by a well-known interferon inducer, polyinosinic–polycytidylic acid (poly I:C); however, the treatment did not clear the infection. These authors continued to show that Mx induction was very high in liver tissue of post-smolts (coincident with the onset of IPNV-induced mortality) as compared with parr, which remained healthy and with low virus titres (Lockhart *et al.*, 2007). Senegalese sole, in which Mx transcription was induced by poly I:C exhibited reduced birnavirus replication on experimental infection (Fernández-Trujillo *et al.*, 2008a). By immunohistochemistry, Das *et al.* (2007) were able to demonstrate Mx proteins in liver, kidney and gill tissue in IPNV-infected post-smolts that also expressed high interferon gene and Mx gene transcripts. Recent studies have reported on the kinetics of IPNV infection and the effects that this exerts on multiple salmonid immune and cytokine genes, demonstrating a number of upregulatory effects (Table 1.9).

It should also be noted that IPNV alone and IPNV–IHNV co-infection experiments in rainbow trout yielded strong Mx expression in kidney, liver and spleen, and fish

Table 1.9. Kinetics of IPNV infection on multiple immune and cytokine genes in salmonid fish.

Fish	Route of IPNV challenge	Tissues	Number of genes assayed	Upregulated genes	Reference
Atlantic salmon	i.p.	Kidney	6	Mx, IFN type 1 and II, γIP	McBeath *et al.* (2007)
Atlantic salmon	i.p.; cohab	HK, spleen, liver, gill	10	Mx, IFN-α, IFN-γ, IL-10, CD8-α, TCR-α, mIgM, MHC-I	Ingerslev *et al.* (2009)
Rainbow trout	i.p.	Spleen, kidney	9	CK-6, CK-7A, CK-5B, CK-1	Montero *et al.* (2009)

that had been stimulated with poly I:C 7 days before showed reduced mortality to both viruses (Rodriguez Saint-Jean and Perez-Prieto, 2007). Trout initially infected with IPNV were also shown to be refractory to experimental infection with IHNV 4 days later (Byrne *et al.*, 2008).

Consequences of persistent IPNV infection

The suppressive effects of persistent IPNV infection on *in vitro* immune parameters have not been well confirmed by *in vivo* protection studies. When experimentally immunizing 2–4 g Atlantic salmon fry against enteric redmouth disease (ERM), Bruno and Munro (1989) found no impairment of the protection in IPNV carriers versus non-carrier fish, as determined by experimental challenge. A somewhat different picture was seen when studying heterologous viral superinfections in IPNV-carrier salmon. When exposed to infectious salmon anaemia (ISA) virus, the mortality of non-IPNV infected post-smolts was consistently higher than in fish that had been IPNV challenged 3 weeks earlier (Johansen and Sommer, 2001). Upon ISAV challenge 6 weeks post-IPNV infection there was, however, no difference between IPNV carriers and non-carriers. In the same paper, the authors reported a similar short-lived protective effect of subclinical IPNV infection against *Vibrio salmonicida* and hypothesized that these non-specific effects could be due to IPNV-induced interferon production. Johansen *et al.* (2009) continued to study persistently infected Atlantic salmon parr over a 10-month period, following common vaccination and sea transfer practices. Furunculosis and coldwater vibriosis challenge experiments were performed. No significant effects associated with i.p. vaccination using a trivalent oil-adjuvanted bacterin lacking IPNV antigen or with smolting were observed in the IPNV carrier group versus non-carrier controls. However, the IPNV-carrier fish experienced a moderate IPN outbreak which produced 24% cumulative mortality in the unvaccinated versus 7% in the bacterin vaccinated fraction. No difference was seen in mortality between immunized IPNV carriers and non-carriers after experimental furunculosis or coldwater vibriosis challenges or in their humoral antibody response to *A. salmonicida* (Johansen *et al.*, 2009). Considering that 10–15 years of field vaccinating IPNV carrier pre-smolts against these diseases have elapsed in Norwegian and Scottish salmon farming, there are almost no reports on bacterial vaccine breakthroughs. In conclusion, and in contrast to infectious bursal disease virus (IBDV) of chicken, IPNV carriage appears in itself not to immunocompromise salmonids to any notable degree.

As to other possible effects, Smail *et al.* (1986) found only faint or non-significant effects of IPNV carriage on growth of Atlantic salmon parr or post-smolts and Damsgård *et al.* (1998) reported that both feed intake and specific growth rate (SGR) of healthy post-smolts were weakly impaired from day 20 through day 45 post-bath challenge with IPNV. Using zebrafish (*Danio rerio*) as a model, LaPatra *et al.* (2000) were able to demonstrate that IPNV infection exerted a strong but relatively short-lived toxic effect on haematopoietic precursor cells in kidney and blood, without resulting in notable changes in haematocrit. This aspect of IPNV infection apparently has not been studied in salmonids.

Induction of clinical outbreaks and predisposing factors

Numerous research groups have tried to identify which host, viral, environmental or husbandry factors determine if IPNV infection will result in an outbreak of overt disease and mortality. The most consistent finding is that fry of all salmonid species are most susceptible shortly after first feeding, thereafter becoming progressively refractory as they approach 4–6 months of age (Frantsi and Savan, 1971; Dorson and Torchy, 1981; Lapierre *et al.*, 1986; Taksdal

et al., 1997). In Atlantic salmon parr and pre-smolts, IPN was for a long time more or less impossible to induce by whatever means (Bowden *et al.*, 2002; Rønneseth *et al.*, 2007). Another consistent finding is the temperature dependence of clinical IPN in salmonid fry. Several authors reported that clinical disease and mortality were most vigorous at water temperatures around 10°C and clearly reduced at 5°C or at 15°C or above (Frantsi and Savan, 1971; Dorson and Torchy, 1981; Lapierre *et al.*, 1986, Okamoto *et al.*, 1987a).

Okamoto *et al.* (1987b) demonstrated that waterborne exposure to increasing concentrations of virulent IPNV gradually gave more rapid onset and higher cumulative mortality in rainbow trout fry, pointing out the role of virus concentration in the induction of disease. In another elegant series of publications, it was shown that the induction of overt IPN corresponded better to a 'point-source' than to a propagative epidemic model, suggesting that induction of disease occurred more or less simultaneously in all animals rather than as a conventional spread from animal to animal (Smith *et al.*, 2000). Both the number of infectious (virus-shedding) fish and the biomass density were associated with a more vigorous course of disease; however, the latter factor diminished with the increasing number of infectious individuals added to susceptible groups (Bebak-Williams *et al.*, 2002). It was further shown that mortality was not induced before virus concentration in the water exceeded 10^3 to 10^5 pfu/l (Bebak and McAllister, 2009). It is easy to imagine that this threshold can be reached without difficulty in modern salmonid aquaculture, given the substantial IPNV shedding from even clinically healthy fish (Urquhart *et al.*, 2008). The principles revealed in this work, in particular the role of group size and number of infectious shedders, may also explain why there is no plain association between the severity of IPNV epizootics in post-smolts and fish density (Bowden *et al.*, 2003).

A reproducible experimental infection model for clinically overt IPN in Atlantic salmon parr or post-smolts has been lacking for many years (Biering *et al.*, 2005), and numerous workers have been frustrated over their inability to induce disease predictably by IPNV inoculation or stress. Several reports have documented the failure of inoculation procedures in various salmonid species beyond the fry stage (Bootland *et al.*, 1986; Rimstad *et al.*, 1991) and numerous unpublished attempts have been unsuccessful in achieving more than faint mortality (P.J. Midtlyng, personal observation). This has been despite the co-administration of cortisone to depress immune responses, which has also failed in striped bass (*Morone saxatilis*) (Wechsler *et al.*, 1986) and brook trout (McAllister *et al.*, 1994). Stangeland *et al.* (1996) and Taksdal *et al.* (1998) finally succeeded in experimentally triggering IPN outbreaks in Atlantic salmon post-smolts, subsequently being repeated by other workers (Bowden *et al.*, 2002; Ramstad *et al.*, 2007). Attempts to correlate IPN mortality with suboptimal seawater adaptation and timing of sea entry were negative (Eggset *et al.*, 1997), and only moderate differences in mortality after IPNV challenge were noted in smolts subjected to hyperoxygenation and low water flow (mimicking intensive rearing systems) as opposed to normoxic fish (Fridell *et al.*, 2007). Hyperoxygenated fish showed cortisol stress responses and increased intestinal permeability in fresh water, which both increased after seawater transfer, coinciding with post-challenge IPNV infection and alterations of the anterior gut mucosa. However, the differences between the oxygenation groups were quite moderate (Sundh *et al.*, 2009).

In summary, it appears that even in genetically susceptible fish (see discussion below), infection via the water (i.e. per immersion or by adding inoculated fish to excrete virus) is required to obtain a 'classical' IPN outbreak pattern. The reasons for this remain unknown, but it is tempting to speculate that specific opportunities for entry and initial replication of the virus in gill and/or gut mucus (and enabling the virus to escape from macrophage sequestering)

may offer an explanation, rather than stress-mediated immunosuppression.

Transmission and Epidemiology

IPN was first suspected to have occurred after the introduction of infected fish or eyed eggs (Snieszko *et al.*, 1957) and this remains the dominant route of IPNV distribution in aquaculture operations. The ability of the IPN virus to persist in clinically healthy hosts has therefore attracted much scientific attention and has been reviewed thoroughly (Wolf, 1988; Reno, 1999; Gregory *et al.*, 2003). Early studies indicated that survivors (healthy carriers) from IPN epizootics could carry the virus for up to 6 years (Wolf, 1966; Yamamoto, 1975b). Many subsequent investigations have confirmed the longevity of IPNV infection but with highly variable prevalence, depending on species, time since the outbreak, population density and diagnostic method. The virus is able to escape the neutralizing activity of circulating antibodies effectively (Ahne and Thomsen, 1986; Bootland *et al.*, 1995) and this is believed to be due to the ability of the virus to infect blood leucocytes (Swanson and Gillespie, 1982) and to replicate in them (Yu *et al.*, 1982). Whether or not individual fish are able to clear themselves completely from IPNV infection cannot be answered with certainty, but there are indications that viable IPN virus is actually able to persist in most, if not all, infected individuals (Munro *et al.*, 2006). There is little doubt, however, that IPNV infection can persist for years at the population level, despite the absence of clinical signs (Murray, 2006). Incomplete development of immunity combined with continuous shedding of virus from some proportion of infected animals presents a mechanism for long-term virus maintenance and prevents a definite clearance of infection. Consequently, there is an inherent and substantial risk of spreading the virus whenever fish from a population with a history of IPNV infection are involved.

Even outside a living host, IPNV shows remarkable features that explain its transmission capacity. Large amounts of virus are shed from infected fish; Billi and Wolf (1969) reported concentrations as high as $10^{7.5}$ infectious doses/ml in faeces from carrier brook trout, and Desautels and MacKelvie (1975) found $>1\times10^5$ $TCID_{50}$/ml in water taken from troughs with rainbow trout fry undergoing an IPN epizootic. McAllister and Bebak (1997) measured up to 1.3×10^4 pfu IPNV/l of water taken near hatchery effluents and were able to isolate IPNV at least 19.3 km below the point of discharge. In a comprehensive work by Urquhart *et al.* (2008), the minimum concentration of IPN virus required to infect Atlantic salmon post-smolts via the water was estimated to be <1 $TCID_{50}$/ml, with the shedding rate at 6.8×10^3 $TCID_{50}$/kg biomass/h. This confirmed earlier information from Smith *et al.* (2000), who were able to induce IPNV epizootics by adding one single experimentally infected animal to groups of naiive rainbow trout fry. Clinical IPN failed to develop in a few of the tanks but nevertheless, IPNV was re-isolated from occasional mortalities, even though the virus titre of the water was <1 pfu/ml. IPNV infection therefore is effectively spread in water reuse or recirculation systems and can spread over long distances with water currents.

Table 1.10. Survival of IPNV in potential vector organisms.

Potential vector	Reference
Wild fish (brown trout)	Munro *et al.* (1976)
Piscivorous birds	Eskildsen and Vestergård-Jørgensen (1973); McAllister and Owens (1992)
Mink	Sonstegard and McDermott (1972)
Cow (ingesting fish silage)	Smail *et al.* (1993a)
Noble crayfish	Halder and Ahne (1988)
Shellfish and marine crustaceans	Mortensen (1993)

The potential of the virus to survive in a diversity of wildlife vectors (some of which are potential scavengers on moribund or dead fish from aquaculture facilities) has been documented (Table 1.10) and must be accounted for in the control of horizontal IPN transmission. Studies of IPN in wild marine fish populations have generally yielded very low prevalence estimates (<1%), with only slight increase patterns in the vicinity of IPN-affected salmonid farms (Wallace *et al.*, 2008).

Vertical transmission

The vertical transmission of IPN virus has received much attention and has been reviewed recently by Bovo *et al.* (2005a). While there is strong evidence that it can occur readily, especially if preventive measures are omitted, there is no clear consensus on the detailed mechanism. Wolf *et al.* (1963) demonstrated high concentrations of infectious virus in ovarian fluids and observed that the virus remained associated with the ova even after washing. The isolation of IPNV from seminal fluid of rainbow trout was reported by Ahne (1983). The conditions of vertical transmission were investigated intensively by several groups (Bullock *et al.*, 1976; Ahne and Negele, 1985). Experimental evidence for the intraovum transmission of IPNV was provided by Dorson and Torchy (1985), who pre-incubated rainbow trout spermatozoa with relatively high concentrations of IPNV before fertilizing eggs from IPNV-free females. IPN developed in all groups of fry originating from eggs that had been fertilized with experimentally infected milt, even when iodophor was used during water hardening (Dorson *et al.*, 1997). It should be noted, however, that other experiments to transmit IPNV vertically have been unsuccessful (Smail and Munro, 1989). Bootland *et al.* (1991) spawned experimentally IPNV-infected brook trout and thereby transmitted IPNV infection to the resultant offspring. Despite numerous experimental and observational studies, it remains unclear whether vertical transmission of IPNV occurs as embryonal infection (virus entering the egg either during oogenesis or attached to the sperm), through contaminated water entering the perivitelline space during hardening, or plainly being rendered inaccessible to iodophor disinfection in pores of the egg shell. Although vertical transmission events appear comparably infrequent compared with horizontal means of IPN virus transmission, this pathway is of major epizootic importance because it provides the biological mechanisms for bridging the infection gap between year classes, for geographical spread with the trade of eyed eggs and for recurrence of IPNV epidemics following eradication measures in hatcheries.

Epizootiological studies

Descriptive epidemiological studies of IPN virus demonstrate two main and distinct scenarios:

1. 'The steady-state scenario' where IPNV is mostly undetectable or is found at a low prevalence in populations where virus titres are low and where outbreaks of disease are absent or rare. Such findings typically come from investigations of wild fish populations such as freshwater resident juveniles or returning spawning fish, and even of fry or fingerlings in stock enhancement hatcheries (Table 1.11).

2. 'The epidemic spread scenario' where virus is readily isolated, with variable but often high prevalence levels, and where virus titres in single fish may reach above 10^5 infectious particles/g of sample tissue, even without clinical signs (Table 1.12). This scenario is being reported predominantly from highly industrialized, commercial fish farming and involves disease epizootics causing mortality and quite severe economic losses.

The scarcity of IPNV in migrating wild salmonids, despite the widespread distribution of IPN virus in farmed salmon along the entire western coast of Norway over many years (Brun, 2003), is remarkable. In our opinion, this pattern suggests that the epidemiological spread of IPNV is determined

Table 1.11. Some recent descriptive epidemiological studies of aquaculture or fish stocks showing low prevalence or incidence of IPNV.

Study area	Fish/farming segment surveyed	Proportion IPNV+ (%)	References
Ontario, Canada	Stock enhancement and commercial sites, freshwater	0–3.0	Bruneau *et al.* (1999)
Switzerland	Stock enhancement and commercial sites, freshwater	0–1.1	Knuesel *et al.* (2003)
West Virginia, USA	Stock enhancement and commercial sites, freshwater	6–20	Bebak-Williams *et al.* (2005)
Norway	Returning wild Atlantic salmon spawners	0–1	Brun (2003)
North-west Pacific USA/Canada	Migratory Pacific salmon juveniles	0	Arkoosh *et al.* (2004)

Table 1.12. Some recent descriptive epizootiological studies on the prevalence of IPNV in industrial salmonid aquaculture.

		Proportion IPNV+ (%)		
Locality	Fish	Freshwater	Marine	References
Spain	Rainbow trout samples	19.5–56	–	Rodriguez Saint-Jean *et al.* (1994)
Norway	Atlantic salmon sites	27.5	35–78	Melby *et al.* (1991); Jarp and Melby (1997)
Scotland	Atlantic salmon sites	5.5–35	31–77	Murray *et al.* (2003)
Ireland	Atlantic salmon sites	0–29	0–56	Ruane *et al.* (2009b)
Scotland	Atlantic salmon broodstock	0.7–9.4	–	Munro *et al.* (2010)

more by the size and density, movements and management of host populations than by the characteristics of the virus.

The course and dynamics of IPNV spread in commercial Atlantic salmon farming described from Norway (Krogsrud *et al.*, 1989; Jarp *et al.*, 1994; Jarp and Melby, 1997), from Scotland (Murray *et al.*, 2003; Bruno, 2004; Murray, 2006) and Ireland (Ruane *et al.*, 2009b) are essentially similar, despite having occurred at different times. Underlying factors in the industrialized salmonid production system, such as high population numbers and densities at all stages of the production cycle, frequent grading and mixing of groups, and trade and transfer of subclinically infected fish, has obviously led to the lack of – or loss of – zoo-sanitary control. In an epidemic scenario, the within-group prevalence of IPNV in hatcheries tends to increase with age and size of the fish (Jarp *et al.*, 1996), while prevalence and titres of detectable IPNV post-sea transfer are maintained or slowly decreasing (Smail and Munro, 1985). In spawning fish, the prevalence of detectable IPNV is often surprisingly low, even in groups that have shown high titres previously in their life cycles (Munro and Ellis, 2008).

In commercial Atlantic salmon farming, clinical outbreaks of IPN have become quite frequent and cumulative incidences of 14% (1993) and 39% (1997–1998) were reported from two Norwegian surveys in freshwater sites (Jarp, 1999). Even higher incidences of clinical disease were reported from post-smolt surveys, ranking from 40 to 70%/year (Jarp *et al.*, 1994; Brun, 2003). In Scotland, the cumulative incidence of clinical IPN in marine salmon farming sites reached nearly 15% in 2002 (Bruno, 2004). Jarp (1999)

identified IPN outbreaks in earlier year classes as a significant risk factor for IPN outbreaks in start-feeding fry, being largest in the first subsequent year. Also, the use of seawater for improving freshwater quality in the hatcheries represented a significant risk. After sea transfer, Jarp *et al.* (1994) found that increased risks of IPN outbreaks were associated with multiple suppliers of smolts and with age of the site (previous period of continuous rearing), both indications that outbreaks might be caused by unknowingly introducing unexposed groups alongside IPNV carriers. Multiple suppliers of smolts were corroborated as a risk factor for IPN in Scotland (Murray, 2006), as was a history of IPN on the site. Murray (2006) postulated that reducing IPNV spread in the freshwater phase, and in particular refraining from multi-origin smolt supplies to marine sites, would contribute most strongly towards control of IPN in marine salmon farming. The same conclusion was made using this model with recent data from Ireland (Ruane *et al.*, 2009b). We agree that reducing IPN infection in first-feeding fry, fingerlings and parr could play a key role in reducing outbreaks and losses due to IPN in commercial salmonid farming.

Control

Zoo-sanitary and health management measures have for decades been the predominant strategies for the control of IPN. Even though these measures have been successful in certain segments of salmonid aquaculture, the disease continues to affect salmonid farming in many countries (e.g. Norway, Scotland and Chile). Notable progress has been made in the past 15 years when it comes to vaccination against IPN-induced mortality. As discussed below, genetic selection for innate resistance to IPN has become a reality and this approach may soon lead to a major leap in the control of this disease.

Zoo-sanitary measures

The primary aim of zoo-sanitary strategies is to block horizontal and vertical transmission between groups of fish through a number of management and biosecurity measures. These strategies include controls on the movement of IPNV-infected fish, separation between rearing units (in space and/or time), testing and exclusion of progeny from IPNV-positive broodfish from the rearing cycle, and disinfection and decontamination procedures. Typically, these measures have been implemented through national or state fish health legislation. However, due to the widespread distribution of the virus and high costs of control, many government agencies have refrained from legal action that can prevent the circulation of IPN virus effectively (Krogsrud *et al.*, 1989; Hill, 1992; Stagg, 2003). This has left essential elements of zoo-sanitary control unrealized or at best subject to voluntary implementation in daily farming operations and fish health management routines.

Fish movement controls and the carrier state

Numerous studies show that in populations without clinical disease signs, the prevalence of cell culture positive IPNV carriers may vary from less than 1% to 100% (Reno, 1999). Billi and Wolf (1969) showed that the amount of virus shed from individual brook trout carriers might fluctuate considerably over time, and other workers (Smail and Munro, 1985; Jarp *et al.*, 1996) have corroborated that virus prevalence tends to decrease post-sea transfer of Atlantic salmon. This feature of IPNV infection thus poses obvious challenges to population diagnosis and health certification, which are key elements of any system to avoid the movement of infected stocks. Schlotfeldt (1979) pointed out that, as a consequence, periodic surveillance and diagnostic activities over several year classes of fish, supplemented with strict biosecurity measures on water supply, vectors, personnel and equipment, were necessary to substantiate that a stock was free of IPN virus. The experiences with movement controls of IPNV also motivated de Kinkelin and Hedrick (1991) to suggest that it was the virus not the clinical disease that needed to be regulated and that

health certificates of fish must relate to the site of origin rather than to the fish lot. For pure statistical reasons, a prevalence of up to 4.9% IPNV carriers may be missed using a random sample of as many as 60 fish, even if the diagnostic test sensitivity is 100%. Consequently, determining the absence of IPNV is more or less impossible based on the outcome of one round of testing.

Separation between fish groups

High concentrations of the virus that are shed from fish groups during IPN outbreaks, the low amount of virus required to establish infection and the remarkable resistance of IPNV to most disinfectants render an effective sanitary separation between tanks or raceways in the same hatchery or freshwater farm very difficult. Efficient sanitary separation between units therefore requires separate buildings, or otherwise spatially separated sections, and, in addition, stringent hygiene measures to avoid virus spread by fomites or vectors. Sanitation after an IPN outbreak must include complete removal of all exposed fish groups in the facility, thorough cleaning and appropriate disinfection and a 'dry' resting time before being re-stocked with specific pathogen-free fish (Schlotfeldt, 1979). This will ensure the separation between susceptible populations on the timescale, and we recommend that freshwater and seawater rearing units that have experienced clinical IPN adopt an 'all-in–all-out' stocking policy to ensure that horizontal transmission and recurrence risks are minimized. Due to its viability under normal environmental temperatures (Ahne, 1982), IPNV can transmit over quite long distances via water. Based on an interesting pathogen dispersion model, Murray *et al.* (2005) suggested that residual current speeds as low as 2 cm/s could transport IPN virus 10–30 km beyond the tidal excursion zone at concentrations exceeding the minimum infectious dose. This allows transmission of IPNV between seawater sites and may, in part, explain the increase in IPNV prevalence observed in Scotland over recent years (Murray *et al.*, 2005), as well as the epizootiology of IPNV in Norwegian marine salmon farms. In conclusion, these factors often limit the option for spatial separation to control horizontal spread of IPNV.

Antibodies as a sign of prior exposure

The presence of anti-IPNV antibodies in carrier rainbow trout was first reported by Wolf *et al.* (1963) and further studied by Wolf and Quimby (1969). Numerous workers have since confirmed that post-infection antibodies tend to persist for a long time in various salmonid species, along with detectable virus (Table 1.13).

It is worthwhile to note that Yamamoto (1975a) showed that the prevalence of antibody-positive rainbow trout increased from 23% in the cold season (late winter) to 40% after the summer. Smail and Munro (1985) compared antibody neutralizing titre against viral titre from two farmed populations of persistently infected Atlantic salmon post-smolts and found no significant positive or negative correlation between the two. This finding has been confirmed (Bootland *et al.*, 1991; Dixon, 1996; Dixon and de Groot, 1996) and it appears that there is no consistent association between the presence of detectable antibodies and the presence or absence of IPN virus in the kidney or gonads of individual fish. As a consequence, serology testing cannot be recommended for segregation of virus-positive from virus-negative parents. Dixon and de Groot (1996) concluded that although the antibody (ELISA) did not detect an antibody response to IPNV in all virus-positive fish, the method had potential for reliably identifying rainbow trout populations previously exposed to IPNV. Thus, antibody tests potentially can contribute cost-effectively to monitor the absence of IPNV infection on a population basis. Avoiding the release of IPNV-infected groups for stock enhancement or the introduction of IPNV-negative smolts to seawater farming sites harbouring IPNV carriers is a key issue for successful zoo-sanitary control of horizontal transmission. Serology

Table 1.13. Reports showing the prevalence of detectable virus and antibodies in IPNV carrier populations of rainbow trout and Atlantic salmon.

Fish	Ab prevalence	Method	IPNV prevalence	References
Rainbow trout	18/45 (40%)	NT	18/18 (100%)	Yamamoto (1975a)
Rainbow trout	38/42 (90%)	NT	23/42 (55%)	Mangunwiryo and Agius (1988)
Rainbow trout	22/44 (50%)	PNT	9/44 (25%)	Dixon and de Groot (1996)
Rainbow trout	24/44 (55%)	ELISA	9/44 (25%)	Dixon and de Groot (1996)
Rainbow trout	40/51 (78%)	ELISA	15/51 (29%)	Dixon and de Groot (1996)
Rainbow trout	5/50 (10%)	ELISA	6/50 (12%)	Dixon (1996)
Atlantic salmon	50/143 (35%)	NT	All (100%)	Smail and Munro (1985)
Atlantic salmon	39/103 (38%)	ELISA	42/103 (41%)	Melby and Falk (1995)
Atlantic salmon	18/29 groups (62.1%)	ELISA	N.d.	Jarp *et al.* (1996)

N.d., not detected; NT, neutralization test; PNT, plaque neutralization test.

would also lend itself well to cost-effective monitoring of the absence of silent IPNV infection in spawning fish. Except for the above-mentioned publications (Dixon, 1996; Dixon and de Groot, 1996), we have found no reports on the application of IPNV serology for such purposes. To what degree serology can give a more reliable indication of prior exposure than virus detection remains a subject of dispute, but we encourage the use of serology to document the long-term specific pathogen-free (SPS) status of broodstock sites and operations.

Broodfish testing and control of vertical transmission

Systematic IPNV testing and subsequent exclusion of progeny from IPNV-positive spawning fish was first proposed by Wolf *et al.* (1968) to control vertical transmission of the disease, and numerous studies have been published on the development and sophistication of this strategy. The recommended method for IPNV surveillance is using tissue culture (inoculation with homogenized liver, kidney or spleen, or ovarian fluid from broodfish on to BF-2, CHSE-214 or RTG-2) with confirmation of cytopathic effects (CPE) using immunological or molecular methods (Anon., 2006). However, a broodstock testing series involving 685 Atlantic salmon from the Faeroe Islands and 103 rainbow trout from Danish fish farms yielded 48% (range 20–100%) positive fish on BF-2 cells but 90% (range 77–100%) positive fish on CHSE-214 cells, indicating that CHSE-214 cells exhibited a significantly higher sensitivity. The data also demonstrated that a combination of the two cell lines could increase IPNV detection by 10%, which might be important for broodstock testing where high sensitivity of detection in single fish is crucial (Olesen *et al.*, 2005). In a study by Munro *et al.* (2010) summarizing the outcome of 12 years of broodstock testing for IPNV in Scotland, it was shown that neither age (1, 2 or 3 sea-winters), geographical location of the broodstock site, nor gender of the fish was associated significantly with prevalence of detectable IPNV.

Pooling of samples has generally been accepted in screening programmes to document freedom from viral infections in populations (Anon., 2006). There is, however, uncertainty as to the degree of interfering factors that may be present in single fish (for example, neutralizing antibodies). A clarification of this potential source of bias for the testing of individual spawners and certification of eyed eggs is urgently required. While experiments have shown that disinfection of IPNV-contaminated eggs is insufficient to block vertical transmission, McAllister (2002) evaluated a procedure for processing sperm that would reduce the IPNV titres in gamete preparations from $\geq 10^6$ pfu/ml to

undetectable levels. This method may allow the recovery of endangered salmonid strains of particular genetic value, should such populations become IPNV infected.

Tissue culture methods are considered very sensitive provided sampling and shipment for laboratory testing are done properly, but the methods require incubation for at least 2 weeks before a negative result can be confirmed. In zoo-sanitary control, this time factor is especially important because of the impracticality and cost of incubating single egg batches in quarantine until the outcome is known. Therefore, there is a need for a rapid, real-time (RT)-PCR method for the detection of IPN virus from reproductive fluids and/or organ tissue of spawning fish. The same is valid for the loop-mediated isothermic amplification (LAMP)-PCR technique that can be carried out without expensive equipment (Soliman *et al.*, 2009). In addition, broodstock often represents very valuable individuals; therefore, the development of any method allowing for non-lethal sampling for IPN screening would be beneficial.

Studies comparing molecular detection and cell culture methods for IPNV in carrier broodfish are scarce. An interesting but rather discouraging result was obtained in an informal ring-test carried out by a major Norwegian producer of salmon eyed eggs. Submitting frozen kidney tissue from 100 female broodfish to three diagnostic laboratories for analysis using conventional RT-PCR and/or cell culture, no fish proved positive on cell culture, whereas 30 samples were positive on RT-PCR. Very surprisingly, not a single fish was unanimously RT-PCR positive in all three laboratories, while only four fish were RT-PCR positive in two out of the three laboratories (Storset *et al.*, 2006). The authors therefore concluded that the RT-PCR testing gave little guidance as to which fish were truly infected and which batch of eggs should be discarded.

Kidney or reproductive fluids for testing?

IPN virus was first detected in ovarian fluid by Wolf *et al.* (1963) and in seminal fluid of carrier salmonids by Ahne (1983). Smail and Munro (1985) compared viral isolation from kidney and ovarian fluid of 40 Atlantic salmon broodfish sampled at a site with a history of IPNV. The results showed that 15 fish were positive on both tissues, whereas 11 fish were positive only in kidney and 6 fish were positive only in the ovarian fluid. Enhanced cell culture isolation of IPNV from the pelleted cell component of ovarian fluid from brook trout was described by McAllister *et al.* (1987) and, in the outcome of subsequent comprehensive work (McAllister *et al.*, 1993), the cell fraction of ovarian fluid and kidney samples gave an equal proportion of isolations by cell culture. Recent data from aquaculture industry programmes suggest that testing of kidney tissue tends to yield a slightly higher proportion of carriers than testing of the sexual products. In a Chilean cell culture screening programme including 150 Atlantic salmon broodfish of each gender, the IPNV prevalence in females was 2% in kidney samples versus zero in ovarian fluid samples. In male fish, the prevalence was 2.8% in kidney samples versus 0.7% in the seminal fluid samples (Olesen *et al.*, 2005). Unpublished data from Scotland using cell culture support this finding, as 153 fish were all IPNV-negative using samples of ovarian fluid cell pellet, whereas 88 were positive in the kidney samples (Eann Munro, personal observation). It should be noted, however, that very few of the comparative studies on IPNV testing methods for broodfish have actually evaluated the merit of either tissue to predict the actual risk for vertical transmission. There are no recent data to show the degree of risk reduction that can be expected by excluding parental fish which are IPNV-positive on either kidney or reproductive fluids. The conclusion of Bootland *et al.* (1991) therefore still appears valid:

> …it was not possible to predict which broodstock carriers would produce IPNV-infected progeny. Failure to detect IPNV in the reproductive products did not ensure that vertical transmission would not occur and, conversely, broodstock carriers with infected reproductive products did not always produce IPNV-infected progeny.

We therefore believe that specific investigations should address these aspects, for example, by studying the vertical transmission rate in progeny from test-positive versus test-negative parental fish.

Success or failure of zoo-sanitary control

Under certain conditions, zoo-sanitary strategies to control IPN can be pursued successfully and certain segments of salmonid culture can be maintained free of IPNV infection for a prolonged period of time. Vestergård-Jørgensen (1977) reported a successful VHS and IPNV control programme in Danish rainbow trout hatcheries, which still operates successfully to maintain IPNV-free (SPF) status. The prerequisite for such success appears to be detailed surveillance and avoidance of using eyed eggs from IPNV-positive spawners, plus strict prohibition of subclinically infected fish to enter the rearing cycle. Examples from Norway, Scotland and Chile, on the other hand, show that IPN has become widespread in industrialized rainbow trout and Atlantic salmon cultures. In Norway, IPN virus was first detected by coincidence in 1975 (Håstein, 1976), but it was not until 1985 that the first clinical outbreak of the disease occurred (Krogsrud *et al.*, 1989). In the meantime, movement restrictions on eggs and fry originating from virus-positive populations with no history of disease had been lifted and mandatory testing of broodfish for IPNV was discontinued. Apparently, similar tactics were followed in Scotland, where IPN standstill notices expired after clinical signs had ceased (Stagg, 2003). In Scotland, statutory requirements for testing of spawning fish from IPNV-positive groups were lifted in 1994 (rainbow trout) and in 2005 (Atlantic salmon).

To summarize, it appears fair to say that if IPN virus (or other birnaviruses) is allowed to circulate in industrialized aquaculture systems, the infection will soon become endemic and eventually pathogenic variants will emerge and cause clinical disease and losses. Conversely, experience shows that in order to be successful, zoo-sanitary control measures for IPN must block both horizontal and vertical transmission pathways and be enforced vigorously.

Inactivation and disinfection

Numerous studies have demonstrated that the IPN virus is remarkably resistant to various physical and chemical disinfection procedures (Table 1.14). In Norway, IPNV has therefore been designated the model test agent for documenting the virucidal effects of aquaculture disinfectants (Anon., 1997). As pointed out by Liltved *et al.* (2006), IPNV is among the most UV-resistant viruses reported in the scientific literature, probably because of its double-stranded RNA. The authors reported that the UV-C dose required for 3-$\log_{10}$ reduction of IPNV infectivity was 246 mJ/cm^2, which was twice the dose required to inactivate a marine nodavirus and more than 30 times the dose required for inactivation of infectious salmon anaemia (ISA) virus. Furthermore, it exceeds the dose reported for UV inactivation of the most resistant human viruses (Liltved *et al.*, 2006). This implies that UV treatments commonly employed for municipal drinking water are not sufficient to inactivate IPN-contaminated hatchery water (influent or effluent). The same authors also demonstrated that substantially higher doses of ozone were required for inactivation of the IPNV in seawater than earlier reported for fresh water. After a total residual oxidant concentration (ROC) equivalent to 7.9 mg Cl_2/l and a contact time of 17 s, the IPN virus titre was reduced by 98.7% (Liltved *et al.*, 2006). IPNV is tolerant to low pH, and conservation of macerated mortalities or offal with acids will retain viable virus even at pH below 3.0 and prolonged contact time (Table 1.14; Smail *et al.*, 1993b). Alkaline treatment reaching a pH near 12.0, however, inactivates the IPN virus quite effectively. It takes hours to inactivate IPNV using heat, unless the temperature is well above 70°C, and results reported by Rapp *et al.* (2000) suggest that 'dry' heat is somewhat less

Table 1.14. Overview of selected inactivation studies with infectious pancreatic necrosis virus (IPNV) (modified after Bovo *et al.*, 2005b).

Method	Dose/concentration	Comment	Contact time	Inactivation	Reference
Heat	60°C		30 min	99.9%	MacKelvie and Desautels (1975)
	60°C		1.5 h	99%	Gosting and Gould (1981)
	60°C	Wet conditions	4 h	Complete	Rapp *et al.* (2000)
	60°C	Dry conditions	4 h	None/minimal	Rapp *et al.* (2000)
	60°C		7 h	99.99%	Gosting and Gould (1981)
	70°C		2 h	Complete	Whipple and Rohovec (1994)
	75°C	Wet and dry conditions	2 h	Complete	Rapp *et al.* (2000)
	80°C		10 min	Complete	Whipple and Rohovec (1994)
	60°C	Fish silage	4 h	>99%	Smail *et al.* (1993b)
UV-C	100–150 mJ/cm^2			99%	Yoshimizu *et al.* (1986)
	120 mJ/cm^2	Freshwater		99.9%	Øye and Rimstad (2001)
	122 mJ/cm^2	Lake, estuarine or seawater		99.9%	Liltved *et al.* (1995)
	150–200 mJ/cm^2	PBS		99.9%	Sako and Sorimachi (1985)
	246 mJ/cm^2	Seawater		99.9%	Liltved *et al.* (2006)
Formalin (formaldehyde)	0.025% (93 mg/l)	4°C, PBS	4 days	99.9%	MacKelvie and Desautels (1975)
	0.2% (740 mg/l)	21°C, aqua dist	1 h	Incomplete	Elliott and Amend (1978)
	2% (7.4 mg/ml)		5 min	Complete	Vestergård-Jørgensen (1973b)
	3% (11.1 mg/ml)		5 min	Complete	Ahne (1982)
Alkaline	pH 11.9	(NaOH)	5 min	Complete	Ahne (1982)
	pH 12.2	8–10°C (NaOH)	10 min	Complete	Vestergård-Jørgensen (1973b)
Acid	pH 2.0	4°C (KCl-HCl buffer)	14 days	99.99%	MacKelvie and Desautels (1975)
	pH 2.5	8–10°C (HCl)	60 min	Minimal	Vestergård-Jørgensen (1973b)
	pH 3	10°C (HCl)	60 min	Incomplete	Ahne (1982)
	pH 4	22°C	14 days	Incomplete	Whipple and Rohovec (1994)
	pH 3.8–4.3	22°C, fish silage	14 days	Incomplete	Whipple and Rohovec (1994)
	pH unknown	4°C, fish silage	5 months	99%	Smail *et al.* (1993b)
	pH unknown	20°C, fish silage	2.5 months	99.99%	Smail *et al.* (1993b)
Propanol	90%	15°C, PBS	90 min	Incomplete	Inouye *et al.* (1990)
Ethanol	90%	15°C, PBS	60 min	Incomplete	Inouye *et al.* (1990)

(*Continued*)

Table 1.14. *Continued*

Method	Dose/concentration	Comment	Contact time	Inactivation	Reference
Methanol	80%	15°C, PBS	30 min	Complete	Inouye *et al.* (1990)
Phenol	5%	15°C, PBS	5 min	Complete	Inouye *et al.* (1990)
Cresol	5%	15°C, PBS	5 min	Complete	Inouye *et al.* (1990)
Iodophor	32 mg/l	PBS	5 min	Complete	Amend and Pietsch (1972)
	35 mg/l	21°C, aqua dist.	5 min	Complete	Desautels and MacKelvie (1975)
	16 mg/l	15°C, PBS	5 min	Complete	Elliott and Amend (1978)
	10 mg/l	15°C, PBS	2.5 min	Complete	Inouye *et al.* (1990)
	2.5 mg/l		60 min	Complete	Inouye *et al.* (1990)
Chlorine	0.1 mg/l	10°C, aqua dist.	1 min	Complete	Wedemeyer *et al.* (1978)
	0.2 mg/l	10°C, soft water	10 min	Complete	Wedemeyer *et al.* (1978)
	0.2 mg/l	10°C, hard water	10 min	Incomplete	Wedemeyer *et al.* (1978)
	0.7 mg/l	10°C, hard water	2 min	Complete	Wedemeyer *et al.* (1978)
	4 mg/l	21°C, titre $10^{4.5}$	5 min	Complete	Elliott and Amend (1978)
	4 mg/l	15°C, PBS	2.5 min	Complete	Inouye *et al.* (1990)
	25 mg/l	20°C, titre $10^{5.0}$	30 min	Complete	Desautels and MacKelvie (1975)
	30 mg/l	10°C, tap water	2 min	99%	Ahne (1982)
	40 mg/l	20°C, titre $10^{7.5}$	30 min	Complete	Desautels and MacKelvie (1975)
Ozone	70 mg/l*h	10°C, soft water	10 min	Complete	Wedemeyer *et al.* (1978)
	90 mg/l*h	10°C, hard water	10 min	Complete	Wedemeyer *et al.* (1978)
	0.11 mg/l (TRO)	Seawater	1 min	99.7%	Itoh *et al.* (1997)
	0.1–0.2 mg/l (TRO)	Lake, estuarine or seawater	30–60 s	99.99%	Liltved *et al.* (1995)
	0.52 mg/l (TRO)	Seawater	1 min	> 99%	Yoshimizu *et al.* (1995)
	7.9 mg/l (TRO)	Seawater	17 s	98.7%	Liltved *et al.* (2006)

efficient than heating under moist conditions. Chlorine remains the most robust chemical for inactivating IPN virus in aqueous solutions, as well as on surfaces (Table 1.14).

Although iodophors are relatively effective against IPNV *in vitro* when applied in concentrations above 10 mg/l (Table 1.14), the detection of IPNV in fertilized salmonid eggs treated with iodophors and in fry hatched from such eggs suggests that infective IPNV is able to escape the commonly applied disinfection procedures. It should therefore be stated explicitly that iodophor disinfection of salmonid eggs does not provide a guarantee against transmission of IPN virus from infected parents to offspring.

In their work to standardize testing procedures for aquaculture disinfectants, Verner-Jeffreys *et al.* (2009) modified the quantitative suspension test methodology recommended by the CEN (Comité Européen de Normalisation) for veterinary disinfectants, using IPNV instead of bovine enterovirus as the test organism. Validation studies were performed with three proprietary chemical products at various concentrations in freshwater suspension, employing a contact time of 30 min at 4°C. The tests showed that all products were able to reduce the amount of cell-culturable IPNV by more than 4-$\log_{10}$ (99.99%) (Table 1.15). The authors concluded that the modified standard (BS EN14675) was well suitable for its purpose. It was noted, however, that one of the products (an acidic iodophor) required further modification (a dialysis procedure) in order to overcome cytotoxic effect of the test product on the cell cultures used for readout.

Vaccination

In his review of IPN vaccination, Dorson (1988) summarized the characteristics of an ideal IPN vaccine, which should:

- protect the fish very early in its life
- allow a rapid onset of protection
- be delivered easily, i.e. orally, or best via the water before feeding starts
- protect against a wide variety of antigenic different strains
- possess the other classical properties of any vaccine (efficacy – innocuousness).

The author found that these prerequisites would indicate a single possible answer – a non-reverting attenuated live vaccine. Twenty years later, we are still waiting for the fulfilment of the desired criteria, and there is no attenuated live vaccine for IPN. However, inactivated IPN vaccines have now become commercially available and are being applied for control of the disease in many segments of commercial aquaculture.

The above-mentioned review paper summarizes the early work on developing IPN vaccines, including rather discouraging results of administering inactivated virus antigen or presumably attenuated strains to young salmonid fry. Key findings of these early trials were that i.p. immunization of fry with formalin-inactivated whole virus vaccine would induce protection against challenge given 2 weeks later, while no protection could be seen after administration of the same antigen orally or via the water (Dorson, 1977). Follow-up studies on alternative techniques for inactivation and treatment of the antigen were negative (Dorson, 1988), both in Europe (Dixon and Hill, 1983b) and in North America (Bootland

Table 1.15. Efficacy of three chemical disinfectants versus IPNV using the modified BS EN 14675:2006 suspension test (contact time 30 min, 4°C) in the presence of interfering substances (yeast extract and BSA). Excerpt from results presented by Verner-Jeffreys *et al.* (2009).

Product	Concentration (v/v) (%)	Control titre ($\log_{10}$)	Titre reduction ($\log_{10}$)
A	0.5	7.41	5.67
B	0.28	6.58	4.17
C	0.033	7.41	≥4.0

et al., 1986). The outcomes of vaccination studies with cell culture attenuated or naturally avirulent live virus were inconsistent and Dorson (1988) therefore concluded that the search for the 'wonder' IPNV strain for immunization had ceased and that inactivated vaccines against IPN would remain too expensive for commercial development, given the fact that the disease problem was confined to fry and young fish.

IPN vaccination of salmon pre-smolts

The emergence of IPN outbreaks in farmed Atlantic salmon smolts and post-smolts (Smail *et al.*, 1992; Jarp *et al.*, 1994) should, however, renew the need for IPN vaccination. Encouraged by results demonstrating the immunogenic properties of IPNV VP2 in rainbow trout fry (Manning and Leong, 1990), Norwegian workers developed a recombinant antigen based on sequences encoding VP2 (rVP2) for inclusion with oil-adjuvanted bacterial vaccines. An injection vaccine with this antigen produced in *Escherichia coli* was launched under provisional marketing authorization in Norway during 1995 (Christie, 1997). To document the vaccine's efficacy, Frost and Ness (1997) immunized 20–30 g Atlantic salmon pre-smolts and challenged them by i.p. inoculation with cell culture-grown IPNV after 10 weeks. The challenge failed, but IPNV was isolated from only two out of 50 fish belonging to the IPN vaccinated group, as compared with 28/50 in saline controls and 16/50 in a control group that had received vaccine without IPNV antigen. The study also showed a rapid and higher antibody response in the vaccinated fish over the controls. Further documentation of the antibody-inducing properties of this vaccine formulation was provided by Biering (1997) and Frost *et al.* (1998). However, the efficacy documentation of this and other IPN vaccines in Atlantic salmon was upheld by the lack of a reproducible challenge model (Biering *et al.*, 2005). After intensive efforts by several research groups, it was shown eventually that mortality could be induced reproducibly in Atlantic salmon smolts and post-smolts through waterborne exposure to virulent IPNV (Bowden *et al.*, 2002), while inoculation systematically failed to induce disease. Using bath and cohabitant challenge, Ramstad *et al.* (2007) finally demonstrated that a commercially available vaccine containing the rVP2 antigen induced approximately 70% IPNV-specific relative protection in Atlantic salmon compared with vaccine lacking this antigen (Table 1.16). The protection estimates obtained experimentally were confirmed through a parallel field trial, where the relative protection of salmon immunized with a vaccine containing rVP2 antigen was 81%.

Based on experience from several years of methodology development, Ramstad and Midtlyng (2008) proceeded to show that the consistency and outcome of IPN vaccine trials in Atlantic salmon was highly dependent on the genetic constitution of the experimental fish. Groups that were susceptible to IPN yielded consistent levels of control mortality and high protection estimates, while both outcomes were much more variable in experimental fish that had been selected for IPN resistance (Fig. 1.8a and b).

Table 1.16. Results (cumulative mortality and relative percentage survival) from experimental and field evaluation of a commercially available IPN vaccine for Atlantic salmon pre-smolts (adapted from Ramstad *et al.*, 2007).

Model	Bath challenge		Cohabitant challenge	Field trial	
Tank/pen number group	L-6	L-7	L-8	Pen 43	Pen 44
Norvax Compact 6 (+IPNV)	16.0%	5.3%	12.8%	6.7%	5.2%
Norvax Compact 4 (–IPNV)	40.3%	29.3%	45.0%	32.4%	30.0%
Control (unvaccinated)	74.7%	78.1%	74.4%	–	–
Relative protection versus controls	85.9%		82.8%	–	
IPN-specific relative protection	69%		72%	81%	

Again, the above-mentioned IPN vaccine gave up to 75–77% relative survival compared with the same formulation lacking IPNV antigen. Other groups have reported moderately protective effects of VP2 subviral particles, produced by recombinant protein technology in yeast (Allnutt *et al.*, 2007).

DNA and oral IPN vaccines

Several constructs for genetic immunization against IPN were evaluated by Mikalsen *et al.* (2004). In a DNA vaccination experiment involving Atlantic salmon smolts that had received various combinations of candidate plasmids, one group (encoding a large segment A polyprotein) showed high relative survival (84%) over the control group, while other constructs were non-significant. To our knowledge, this concept has not been pursued further for licensing. Very recently, a second group has reported very promising data from DNA vaccination and DNA-based immunotherapy trials (de las Heras *et al.*, 2009). Brown trout and rainbow trout of 1–2 g were immunized orally with a vector carrying the VP2 capsid gene, incorporated into alginate microparticles. In experimental immersion challenges

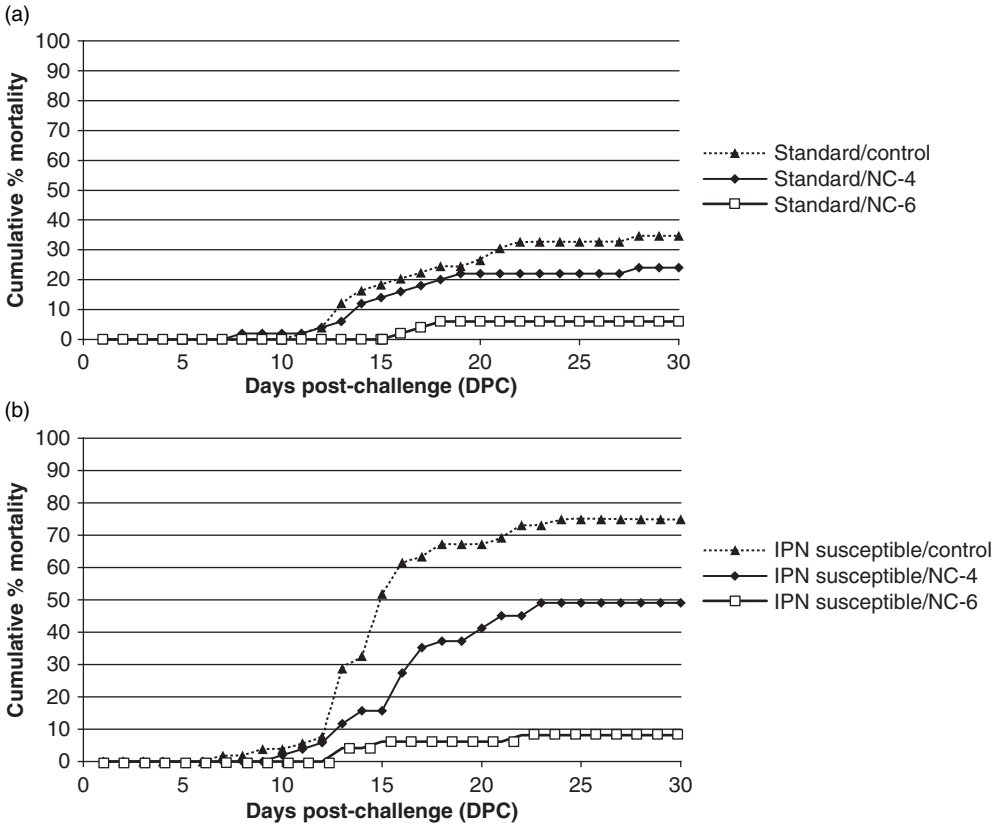


Fig. 1.8. Cumulative mortality curves for Atlantic salmon smolts after bath challenge with IPNV 10 weeks after IPN vaccination: (a) 'standard' fish (parents selected for IPN resistance; (b) IPN-susceptible fish (offspring from deselected parents). NC-6: multivalent vaccine with IPNV antigen; NC-4: multivalent vaccine without IPNV antigen. Control: unvaccinated group. Reproduced from Ramstad and Midtlyng (2008) Strong genetic influence on IPN vaccination-and-challenge trials in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 31, 567–578, with permission from John Wiley and Sons.

carried out 15 or 30 days after vaccine delivery, the DNA-immunized fry showed a high relative survival and lower titres of virus than non-immunized fish.

In 2006, an oral vaccine against IPN was licensed in Chile, recommended for use in healthy Atlantic salmon fry and with a claim to protect against disease for at least 128 days (www.spaquaculture.com/default.aspx?pageid=661; accessed 2 December 2009). According to a study report accessible from the manufacturer's website, the vaccine induced neutralizing antibodies from day 4 and showed good protection (65–88 RPS) in challenge experiments performed after primary immunization and after the first and second boost. Field trials with this vaccine have also been initiated in Norway. Unfortunately, neither the above study nor results from field studies in Chile or Norway have been published in peer review journals, precluding an independent scientific assessment of the results.

Effect of IPNV on postvaccinal abdominal adhesions

The safety of vaccinating IPN-infected fish has been addressed through an experimental study, during which Atlantic salmon presmolts were inoculated with live IPN virus 14 days before they were vaccinated intraperitoneally with a trivalent, oil-adjuvanted bacterin formulation (Colquhoun *et al.*, 1998). Co-infection with IPNV did not affect the severity of abdominal vaccine lesions as determined by necropsy 1, 2 and 3 months after vaccination, while co-injection with live *Pseudomonas fluorescens* and, to a lesser degree, periodical crowding stress did aggravate the abdominal lesions. Based on these and later results (Berg *et al.*, 2006), concurrent IPNV infection has not been considered a risk factor for aggravating side effects when vaccinating Atlantic salmon parr.

Interpretation of epizootiological data

Several epizootiological studies conducted in Norway since 1997 have provided information on IPN vaccination, and both Midtlyng (2003) and Brun (2003) have attempted to analyse quite comprehensively the outcome of IPN vaccination of presmolts in the field. The studies showed that the usage of vaccines for Atlantic salmon containing IPN antigen went up steadily, reaching above 90% in the study area in 2001–2002. However, IPN vaccination did not give a marked reduction in the proportion of sites reporting IPN outbreaks among post-smolts, or an obvious drop in mortality during such outbreaks. It should be noted that due to the crude nature of the data, lack of IPN unvaccinated controls, and fluctuations in the use of specific vaccine brands from one year to another, only limited conclusions can be drawn from these studies. Recent information indicates that there is still close to 100% IPN vaccine coverage in Norwegian salmon farming and that both the IPN outbreak incidence and losses during outbreaks have been reduced in later years (Anon., 2009). Also, in Chile, vaccination against IPN apparently has increased above 60% coverage in Atlantic salmon smolts (Bravo and Midtlyng, 2007) but no field results on its effect are available.

Based on the information to date, it can be summarized that injectable IPN vaccines do provide specific protection against losses during outbreaks of the disease in post-smolts, a feature that merits their use in the control of IPN on purely economic grounds. However, the current vaccines based on inactivated antigens cannot fully prevent clinical outbreaks of the disease or prevent the carrier state, and thus they cannot provide an effective prevention against horizontal or vertical disease transmission of the virus. To develop and document oral and/or immersion vaccines that can protect salmonids effectively during the most susceptible stage of their life cycle, as fry or fingerlings, remains a big challenge in IPN vaccinology.

Treatment

Attempts to find a chemotherapy for IPN have been repeatedly unsuccessful. Only a handful of studies have reported promising outcomes of *in vitro* experiments, and even

fewer papers show *in vivo* effects (Table 1.17). In accordance with the general situation in veterinary medicine, one can therefore say that medicinal treatment to control IPN and other viral infections is far out of sight.

In the recent decade, a number of attempts have been made to control IPN or IPN virus infection by immunostimulation. Siwicki *et al.* (1998) reported that rainbow trout fed a dimerized lysozyme ('KLP-602') experienced reduced overall mortality (30% versus 65%) in a population experiencing an IPN outbreak, but without giving data on the IPN-specific proportion of the losses. In an experimental IPN challenge trial carried out by Johnsen (2002), mortality reached 35% in fish receiving control feed, while only 26% of the Atlantic salmon smolts receiving the same feed supplemented with β-1,3/1,6 glucan plus nucleotides died. Mortality in a second control group receiving a competing feed supplemented with nucleotides reached 38%. In contrast, Leonardi *et al.* (2003) published that nucleotide-enriched feed could protect rainbow trout from mortality induced by inoculation challenge with IPNV. It should be noted, however, that this result was based on a study with only eight fish per group. The scientific weaknesses of the study and the lack of further evidence for successful control suggest that the effects of oral immunostimulants against IPN are limited. Lockhart *et al.* (2004) conducted an interesting experiment in Atlantic salmon growers that were IPNV carriers using poly I:C for treatment. While leucocytes from unstimulated carriers showed no Mx expression, injection of poly I:C strongly induced Mx expression for 7 days. However, IPN virus could still be cultured from the leucocytes of stimulated fish, showing that the treatment had been insufficient to cure the carrier state. When poly I:C was used for immunostimulating Senegalese sole prior to inoculation with a birnavirus from the same species, Fernández-Trujillo *et al.* (2008b) found that replication in kidney cells was suppressed strongly for up to 72 h. To what degree immunostimulatory treatment can render fish refractory to waterborne IPNV infection therefore still remains unknown.

Management interventions to alleviate IPN outbreaks

In the absence of medicinal treatment, both researchers and fish health practitioners have attempted to prevent IPN outbreaks and/or alleviate ongoing outbreaks by various management interventions. Veterinarian Trude Bakke Jøssund (2007) summarized the experiences made with such intervention practices by Marine Harvest Norway, pointing in particular towards two remedial actions that have shown an effect: (i) increase in water temperature following outbreaks; and (ii) vaccination of pre-smolts. According

Table 1.17. Chemical substances reported to suppress IPN virus replication *in vitro* and/or *in vivo*.

	References	
Substance	*In vitro* studies	*In vivo* studies
Iodine		Economon (1963, 1973)
Ribavirin	Migus and Dobos (1980); Jashes *et al.* (1996)	Savan and Dobos (1980)
Halocyamine	Azumi *et al.* (1990)	–
Amantadine	Hudson *et al.* (1988)	–
EICAR	Jashes *et al.* (1996, 2000)	Moya *et al.* (2000)
Pyrazofurin	Jashes *et al.* (1996)	–
Mycophenolic acid	Marroqui *et al.* (2008)	–
Filifolinyl senecionate	Modak *et al.* (2010)	–

to the author, an increase in rearing temperature to 16–18°C for 7–12 days tends to reduce the daily mortality during IPN outbreaks in first-feeding Atlantic salmon fry. There is, however, a risk of recrudescence when temperatures are subsequently reduced again. She also reported several examples that injection vaccination using multivalent, oil-adjuvant bacterins would alleviate IPN outbreaks in parr and pre-smolts, and that vaccination 4 weeks or shorter before would reduce mortality during IPN outbreaks, shortly after sea transfer. While the vaccination effects were attributed to interferon induction, improved immune responses were held responsible for the temperature effects. Also, the improvement of hatchery water through various kinds of buffering treatment was believed to reduce the IPN-induced mortality.

Breeding for IPN resistance

Genetically distinct strains of the same fish species exhibiting different susceptibility to IPN were first reported by Silim *et al.* (1982). In a series of studies of rainbow trout, Okamoto *et al.* (1987c) reported that breeding the survivors from annual IPN epizootics eventually led to the formation of one IPN susceptible (Okamoto *et al.*, 1987d) and one resistant rainbow trout strain (Okamoto *et al.*, 1993), which were used subsequently in the search for molecular genetic markers. Using a framework linkage map based on microsatellite markers, two quantitative trait loci (QTLs) affecting IPN resistance were detected on two different chromosomes, suggesting that IPN resistance was polygenic in rainbow trout (Ozaki *et al.*, 2001). These two loci were responsible for a significant portion (27–34%) of the total phenotypic variation observed during challenge trials. This work has been followed recently by the identification of further QTLs for IPN susceptibility in rainbow trout (Ozaki *et al.*, 2007).

Following breeding efforts to increase resistance to furunculosis and infectious salmon anaemia and a successful pilot evaluation for IPN, a Norwegian breeding company started selection of their Atlantic salmon stock for IPN resistance during 2001 (Midtlyng *et al.*, 2002). In this scheme, the selection of breeders has been based on the performance of their full-sibs and half-sibs after IPNV challenge tests performed at the fry stage. Over a 10-year period, the heritability estimates for resistance to IPNV-induced mortality varied between 0.16 and 0.41 (Table 1.18), and there was good correlation between estimates obtained in experimental challenge and field outbreaks of IPN (Wetten *et al.*, 2007). Challenge

Table 1.18. Summary of heritability estimates for resistance to IPN mortality studied in Norwegian (Wetten *et al.*, 2007) and Scottish (Guy *et al.*, 2009) breeding populations of Atlantic salmon.

Norwegian strain		Scottish strain	
Year class	Heritability estimate (h^2)	Year class	Heritability estimate (h^2)
1997	0.233		
1998	0.265		
1999	0.319		
2000	0.394	2000	0.16–0.28 (2 sites)
2001	0.397	2001	0.56
2002	0.233	2002	0.11–0.41 (3 sites)
2003	0.165	2003	N.d.
2005	0.327	2005	0.07
Overall	0.310 (SE ± 0.060)	Overall	0.38 (SE ± 0.017)

N.d., not detected.

experiments performed with first-feeding fry and at the smolt stage have confirmed the selection response obtained through this programme (Storset *et al.*, 2007).

According to Kjøglum *et al.* (2008), the genetic correlations between resistance to IPN, ISA and furunculosis were weak and non-significant, suggesting that the resistance mechanisms for these diseases were under independent genetic control. Another consequence of this finding was the ability to select for each of the traits without the risk of exerting a major effect on the other.

In parallel to the Norwegian efforts, Scottish workers independently, and in a different breeding strain of Atlantic salmon, found equally strong heritability when analysing IPN outbreaks in pedigreed post-smolts being held at different seawater sites (Guy *et al.*, 2006; Table 1.18). The results were confirmed in later year classes of the same strain by Houston *et al.* (in press).

Genomic marker-assisted selection (MAS) for IPN resistance

A major leap towards improving the genetically determined resistance of salmonids to IPN was made when Houston *et al.* (2008) were able to identify two genomic QTLs for IPN susceptibility or resistance in pedigreed groups of Atlantic salmon following field outbreaks of IPN in post-smolts. Within one family of Atlantic salmon, the difference in cumulative mortality between homozygote offspring carrying or not carrying this marker were 9% versus 88%, respectively. In parallel and independent efforts, Norwegian workers confirmed the genomic location of this QTL and showed that it could explain 29% of the phenotypic and a remarkable 83% of the genotypic variance observed during IPNV challenge experiments (Moen *et al.*, 2009). While the IPN QTL was assigned to the same linkage group on the salmon genome map by both groups, there is as yet no information as to its exact relation to functional genes that could explain the effect. In summary, these results mutually confirm each other and lend credit to the relevance of the finding. The magnitude of the effects for IPN in salmon is indeed hitherto unsurpassed in MAS of animals and suggests that this novel technology holds great promise in the control of IPN in Atlantic salmon farming in the foreseeable future.

Conclusions and Recommendations for Future Studies

The wealth of scientific literature on IPN and other diseases illustrates the enormous resources (intellectual and financial) that are being invested to face the challenges caused by aquatic birnaviruses worldwide. The research focus in recent years has been on the virulence of IPN virus, on host–pathogen interactions, on immune mechanisms and on the heritability of disease resistance. In line with current trends in biomedical and life sciences, the molecular genetic processes have received most attention, in regard to both the pathogen and the host. In several aspects, this basic research has provided novel discoveries and insights, which we hope can soon be converted into the practical prevention and control of disease.

Through the discovery of genomic markers with exceptionally strong predictive power for disease resistance (Houston *et al.*, 2008; Moen *et al.*, 2009), the control of IPN-induced mortality in Atlantic salmon looks very promising, even in a relatively short time perspective. The next challenge is obviously to identify the functional mechanisms responsible for this resistance, which has the potential to stimulate the targeting of other means of disease control such as vaccination or treatment. There is also a need to determine the heritability of IPN or birnavirus disease resistance and to discover these genomic markers in other aquaculture species. In a longer and strategic perspective, the breeding of salmonids that are refractory to IPNV infection is a tempting idea, because potentially it can reduce the health management and disease control problems caused by persistent infection.

We hope that the upcoming discovery of the tri-dimensional structure of the IPNV virion (Coulibaly *et al.*, 2010) will initiate new developments in IPN vaccinology, eventually bringing out novel 'second-generation' vaccines that can provide more effective protection against clinical disease or, even more importantly, early, stable and lasting immunity to IPNV in salmonid fry and fingerlings. The beneficial epizootic effects of effectively being able to immunize first-feeding fry should not be underestimated and both basic research and vaccine development to achieve this goal should be strongly encouraged.

Equally if not even more challenging is further research to unravel the exact mechanisms for vertical transmission of IPNV and other aquatic birnaviruses from infected parental fish to their offspring. This knowledge is needed to find reliable procedures to block vertical transmission and thereby prevent the re-contamination of hatcheries and production cycles. An effective control of vertical transmission will thus encourage the industry to pursue more vigorous IPN control and assist health management and efforts further down the production cycle.

We believe that the composite effects of novel genetic, vaccination and zoo-sanitary methods eventually will enable the effective control of IPN and IPN virus infection in salmonid aquaculture. Given the worldwide distribution of aquatic birnaviruses and the apparent potential of this virus family to evolve with and create problems for the emerging culture of new fish species, further research into solving the IPN problem of salmonids today may also prove a long-term investment for the future.

The authors wish to acknowledge Dr Torunn Taksdal, Patricia Noguera and Prof Øystein Evensen for providing images.

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2 Infectious Haematopoietic Necrosis Virus

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Introduction

Infectious haematopoietic necrosis virus (IHNV) is a rhabdovirus that causes an economically significant disease in Pacific salmon (*Oncorhynchus* spp.), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). The disease was observed initially in cultured sockeye salmon (*O. nerka*) on the west coast of North America (Rucker *et al*., 1953; Watson *et al*., 1954) and IHNV was first isolated in cell culture by Wingfield *et al*. (1969). Typically, IHNV causes necrosis of the haematopoietic tissues. The disease was named infectious haematopoietic necrosis (IHN) by Amend *et al*. (1969). Economic losses from IHNV can be a direct consequence of fish mortality, or indirect from regulations restricting the movement of IHNV infected fish or the destruction of infected fish stocks to control the spread of the virus. Infectious haematopoietic necrosis is the most important constraint to the profitability and continued growth of the US commercial salmonid aquaculture industry. In 2004, outbreaks of IHN disease in Atlantic salmon in British Columbia resulted in an estimated loss of US$40 million, which represented US$200 million in lost sales (Lorenzen and LaPatra, 2005). In addition to the Pacific north-west, IHNV is also endemic in salmonid fish in Japan and has been isolated in several other countries in Asia and Europe (OIE, 2007).

Reviews of early reports of IHN and IHNV have been published (Pilcher and Fryer, 1980; Nicholson, 1982; Wolf, 1988) and this chapter is an update of our 1999 review (Bootland and Leong, 1999).

The Disease Agent

Current classification

Infectious haematopoietic necrosis virus (IHNV) is a fish rhabdovirus and is considered one of the most serious viral pathogens of aquacultured fish. IHNV is the type species of the genus *Novirhabdovirus* (family *Rhabdoviridae*). Other fish rhabdovirus species that belong to this genus include viral haemorrhagic septicaemia virus (VHSV), hirame rhabdovirus (HIRRV), snakehead rhabdovirus (SHRV) and eel virus B12 (EV-B12) and C26 (EV-C26) (Essbauer and Ahne, 2001; Hoffmann *et al*., 2005). All these viruses have ssRNA viral genomes that are present in the virion as negative sense RNA with the following gene order from the 3′-end: leader-*N-P-M-G-NV-L*, where *N* is the nucleoprotein gene, *P* is the phosphoprotein gene, *M* is the matrix

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protein gene, *G* is the glycoprotein gene, *NV* is the non-virion protein gene, and *L* is the virion RNA polymerase gene. The presence of the *NV* gene distinguishes this group of rhabdoviruses in the genus *Novirhabdovirus*, 'Novi' standing for non-virion (Kurath *et al.*, 1997). A comparison of nucleotide and amino acid sequences of the structural proteins also supports the classification of the Novirhabdoviruses as a separate genus of the Rhabdoviridae (Johnson *et al.*, 1999).

Biology

Disease signs

IHNV infection is a serious disease of young salmonid fish, although the virus can infect salmonid fish of all ages. In acute disease, there is a sudden increase in fish mortality, but the fish may not show clinical signs and may die without apparent cause. More typically, at the start of an epizootic, there are moribund fish that are lethargic, with periods of sporadic whirling or hyperactivity. Moribund fry also may have a dark coloration, a distended abdomen, exophthalmia, pale gills and mucoid, opaque faecal casts. Petechial haemorrhage may be observed at the base of the fins and vent, and occasionally in the gills, mouth, eye, skin and muscle. In Chinook salmon (*O. tshawytscha*), but not in sockeye salmon, the fry may have a subdermal haemorrhagic area immediately behind the head (Yasutake, 1970; Amend, 1975). In older fish, there are fewer external clinical signs. Two-year-old kokanee salmon have erratic swimming and haemorrhage near the base of the fins (Traxler, 1986) and sockeye salmon smolts have gill and eye haemorrhage, clubbed and fused lamellae and cutaneous lesions (Burke and Grischkowsky, 1984).

The liver, spleen and kidney of fry are pale due to anaemia; there may be ascites; and the stomach is filled with a milky fluid but without food. The intestine contains a watery, yellowish fluid and there may be petechial haemorrhages in the visceral mesenteries, adipose tissue, swim bladder, peritoneum, meninges and pericardium (Rucker *et al.*, 1953; Ross *et al.*, 1960; Wolf, 1988). Older fish may have empty stomachs, intestines filled with yellowish mucus and lesions in the musculature near the kidney (Traxler, 1986).

Virus strain characterization

Early work on the characterization of different isolates of IHNV with polyclonal antisera indicated that there was only one serotype of the virus (McCain *et al.*, 1971). However, in 1985 Hsu *et al.* (1985, 1986) developed a method of distinguishing different IHNV isolates based on the electrophoretic migration patterns of the viral proteins on SDS-polyacrylamide gels. Five electropherotypes were identified in that study and showed the virus 'type' was not host specific; rather, all species in a geographic region carried the same electropherotype. These findings made it possible to track viral strains newly introduced into a region.

Further verification that there was only one serotype of IHNV was carried out with antisera to the viral glycoprotein (67 kDa). The IHNV glycoprotein had been shown to be the only viral protein capable of eliciting a neutralizing antibody response in rabbits and providing protective immunity in young fish (Engelking and Leong, 1989a,b). Purified glycoprotein from IHNV-RB-1 protected the fish against challenge with a potentially lethal IHNV infection (Engelking and Leong, 1989a). The work was extended to show that the purified glycoprotein from IHNV-RB-1 also induced protective immunity against the five IHNV electropherotypes by immersion vaccination (Engelking and Leong, 1989b).

A comparison of the neutralization indices with ten different isolates of IHNV representing all five electropherotypes against anti-IHNV-RB-1, anti-IHNV-CO-2, anti-G protein of IHNV-RB-1 and anti-G protein of IHNV-CO-2 sera also confirmed that

there was only one serotype. The four antisera did define two serovariant groups: a group of readily neutralized IHNV isolates, including RB-1 (type 1), CO-2 (type 4), Elk River (ER) (type 3), CD-2 (type 5), HA-1 (type 2) and TA-1 (type 1), and a group of less readily neutralized virus isolates, including DW-2 (type 3), DW-3 (type 3), LE (type 2) and NS (type 3).

Monoclonal antibody (MAb) neutralization assays replaced electropherotyping for characterizing isolates of IHNV. Winton *et al.* (1988) separated 12 IHNV strains into four groups, using three MAbs. Further characterization of 12 different IHNV isolates with seven MAbs produced ten different reactivity patterns (Nichol *et al.*, 1995). The atypical IHNV isolate, HO-7, was not neutralized by any of the seven MAbs. Ristow and Arnzen-de Avila (1991) examined 17 isolates of IHNV with a bank of five glycoprotein-reactive MAbs. Twelve of the 17 isolates were neutralized by two MAbs (3GH127B and 3GH92A) and, of the 12, one was the only isolate neutralized by a single MAb (1GH131A). Five isolates were not neutralized by any of the MAbs. Two MAbs did not neutralize any of the virus isolates (3GH135L and 2GH5F). When the same 17 isolates were examined with a bank of 15 MAbs reactive with either the N or G proteins, 15 distinct reaction patterns were observed. These studies supported the conclusions that strains of IHNV were distributed geographically and that different species in a geographical location could harbour the same type of IHNV.

Kurath *et al.* (1995) used RNAse protection assays to evaluate the differences in strains of IHNV, and Oshima *et al.* (1995) used two-dimensional oligonucleotide patterns to assess genetic diversity among different IHNV isolates. All of these techniques have confirmed that IHNV strains are geographically confined and the appearance of a new strain in an area may indicate the introduction of IHNV infected fish or eggs. More recently, sequence analysis of a 303 nucleotide variable region of the IHNV glycoprotein gene for 323 isolates from infected salmonid fish throughout the Pacific north-west of North America revealed three major virus genogroups, designated U, M and L (Garver *et al.*, 2003; Kurath *et al.*, 2003; Troyer and Kurath, 2003). The genogroup U (from Alaska, the British Columbia coastal watershed and the Columbia River), the genogroup M (from the Columbia River and Idaho) and the genogroup L (from California and the southern Oregon coast) were identified. The L genogroup was enlarged recently by the analysis of 237 IHNV isolates primarily from hatchery-reared and wild Chinook salmon in California (Kelley *et al.*, 2007). The same approach was used by Enzmann *et al.* (2005) to demonstrate that the IHNV that first appeared in rainbow trout in Europe in 1987 most likely originated from an ancestor of the M genogroup from North America. In Japan, the IHNV isolates group with the U genogroup, consistent with the reported introduction of IHNV in 1967 via a shipment of contaminated sockeye salmon eggs from Alaska (Nishizawa *et al.*, 2006). A study by Kolodziejek *et al.* (2008) also used the 616 bp long nucleic acid sequence from the middle region of the *G* gene to construct a phylogenetic tree of the IHNV strains in Europe, the USA and Japan. The study uncovered two distinct IHNV clusters among the European isolates (European group 1 and European group 2), and both of these clusters were related more closely to the genogroup M identified by Kurath *et al.* (2003). In Russia, IHNV isolates obtained from sockeye salmon in the Kamchatka Peninsula contained nucleic acid sequences indistinguishable from the sequences of North American U genogroup isolates that occurred throughout Alaska, British Columbia, Washington and Oregon (Rudakova *et al.*, 2007). The high similarity, and in some cases identity, between Russian and North American IHNV isolates suggests viral transmission or exposure to a common viral reservoir in the north.

Ultrastructure

The IHNV virion is bullet-shaped and measures 110 nm × 70 nm in fixed, thin-sectioned

preparations in infected cell cultures and 111 nm×11 nm in negatively stained preparations of purified virus (Hill *et al.*, 1975).

Infectious haematopoietic necrosis virus was one of the first fish rhabdoviruses to be characterized biochemically, and it has five structural proteins (McAllister and Wagner, 1975). The five virion proteins include a high molecular mass L or polymerase protein (150–225.2 kDa), a glycoprotein G (67–70 kDa), a phosphorylated nucleoprotein N (40.5–44 kDa), a phosphorylated P or Ml protein (22.5–27 kDa) and a matrix protein M or M2 (17.5–21.8 kDa). A non-virion protein NV of 12 kDa has also been identified in infected cells (Kurath and Leong, 1985; Schutze *et al.*, 1996).

An estimate of the number of molecules per virion for each protein was made for IHNV (Leong *et al.*, 1983). The ratio of viral protein to ribonucleic acid (RNA) is 21:1, a very low figure in comparison with vesicular stomatitis virus (VSV) and rabies virus (RV), which have ratios of 92:1 and 72:1, respectively (Bishop and Roy, 1972; Coslett *et al.*, 1980). The low protein–RNA ratio for IHNV is unusual and may reflect differences in the membrane structure of fish and mammalian cells (Moore *et al.*, 1976). It is similar to that obtained for La Crosse virus, a bunyavirus, which has a ratio of 30:1 in BHK/21 cells (Obijeski *et al.*, 1976).

The relative contribution of each protein to the total molecular weight of the virion was estimated from densitometer tracings of SDS-polyacrylamide gels of purified virus. The numbers of molecules of IHNV virion proteins are 23–40 L, 198–290 G, 560–774 N, 391–514 P and 874–1044 M. These are strikingly different from those of rabies virus, which are 79 L, 1723 G, 1975 N, 402 P and 1156 M (Coslett *et al.*, 1980). The remarkable difference is in the number of G molecules per virion. It is difficult to produce high-titre neutralizing antibody to IHNV in warm-blooded animals. The poor immunogenicity of G or the poor neutralizing activity of the antisera may be a direct result of the low numbers of G molecules on the surface of the IHN virion.

Transmission

IHNV is primarily transmitted horizontally from fish to fish (Wolf, 1988). Viral titres in water initially may be undetectable or very low, but are amplified by repeated cycles of shedding and infection and may reach over 10^3 $TCID_{50}$/ml during epizootics in young fish (Nishimura *et al.*, 1988; Zhang and Congleton, 1994). After immersion infection, rainbow trout fingerlings may not become infectious or start shedding virus for 2–3 days. Thereafter, a single infected fish is capable of transmitting infection to cohabiting susceptible fish (Ogut and Reno, 2004a). Waterborne IHNV can also result in horizontal transmission within and between species of cohabiting adult salmonids in fresh water (Mulcahy *et al.*, 1983a; Burke and Grischkowsky, 1984; Traxler, 1986; LaPatra *et al.*, 1989a; Kelley *et al.*, 2007) or the marine environment (Traxler *et al.*, 1993; St-Hilaire *et al.*, 2002).

Vertical viral transmission is the transmission of virus from one generation to its offspring, regardless of whether the virus is located within or external to the egg (Pilcher and Fryer, 1980). Vertical transmission of IHNV is based primarily on indirect evidence such as the shipment of infected eggs into new geographical areas, with resultant outbreaks of IHN and occurrence of IHN in progeny from eggs disinfected with iodophor and raised in virus-free water (Sano *et al.*, 1977; Niu and Zhao, 1988; Park *et al.*, 1993; Bootland and Leong, 1999). However, there is doubt whether vertical transmission really occurs since it is very difficult to demonstrate experimentally (Mulcahy and Pascho, 1985) and it may be an infrequent event since eggs can be reared in virus-free water and remain virus-free (Amend, 1975; Engelking *et al.*, 1991; LaPatra *et al.*, 1991; Yamazaki and Motonishi, 1992; Meyers, 1998). Fish species, the stage of egg development and the presence of viral-inhibiting components in the egg may be important determinants for vertical transmission and replication of IHNV (Amend, 1975; Burke and Mulcahy, 1983; Mulcahy and Pascho, 1985; Yoshimizu *et al.*, 1989; Bootland and

Leong, 1999). Vertical transmission of IHNV with survival of some infected embryos that then horizontally transmit virus is thought to be responsible for many hatchery outbreaks in Alaskan sockeye salmon, but clinical disease may not occur until triggered by an environmental stressor (Meyers, 1998). Male salmonid fish may have a role in vertical transmission since IHNV is frequently isolated from milt, but at lower viral titres than in ovarian fluid (Mulcahy *et al.*, 1987; Meyers *et al.*, 1990; Yamazaki and Motonishi, 1992). IHNV strongly and quickly adsorbs on the surface membrane of sperm and although there is no direct evidence, this could deliver the virus directly into the egg during fertilization (Mulcahy and Pascho, 1984; Yoshimizu *et al.*, 1989; Meyers *et al.*, 1990).

Geographical Distribution and Host Range

IHNV has a broad geographic and host range, but primarily causes disease in salmonids. The virus was isolated originally in the Pacific north-west of the USA during the 1950s from sockeye salmon at hatcheries in Washington (Rucker *et al.*, 1953) and Oregon (Wingfield *et al.*, 1969), followed by outbreaks in Chinook salmon in California (Ross *et al.*, 1960). Although sporadic IHN outbreaks have occurred in other states of the USA during the 1970s and 1980s (Bootland and Leong, 1999), IHNV is currently endemic in the Pacific north-west of North America, extending from Alaska to California and inland to Idaho (Kurath *et al.*, 2003). In 1992, IHNV was reported for the first time in British Columbia, Canada, in Atlantic salmon in saltwater net-pen sites (Armstrong *et al.*, 1993). IHNV was spread by the movement of infected eggs and/or fry outside North America (Hill, 1992) and has been identified in several other countries. The virus spread from Alaska to Japan in 1968 (Sano *et al.*, 1977), then from Japan to north-east China (Niu and Zhao, 1988) and Korea (Park *et al.*, 1993). IHNV has also been isolated sporadically in Taiwan (Chen *et al.*, 1985; Wang *et al.*, 1996). The Office International des Epizooties (OIE, 2007) maintains a World Animal Health Information Database (WAHID) that tracks specific animal diseases and, unless otherwise referenced, the geographical information listed below has been obtained from this database. In Europe, IHNV was introduced in 1987 to Italy (Bovo *et al.*, 1987) and France (Baudin-Laurencin, 1987). IHNV was then identified in Germany (Enzmann *et al.*, 1992) and Belgium (Hill, 1992), and also in the 1990s in Austria, the Czech Republic, Poland and the Netherlands. Sporadic or limited occurrences have been reported in Slovenia (Jenčič *et al.*, 2002), Croatia (Vardiç *et al.*, 2007), Iran, Kuwait, Pakistan, Spain and Switzerland (OIE, 2007). IHNV was identified in 2000 in the Russian Federation (Rudakova *et al.*, 2007). In South America, IHNV was identified in Bolivia and suspected but not confirmed in the Dominican Republic (OIE, 2007). Although there appear to be no new countries reporting IHNV from the mid-2000s to present, it is likely that the geographical range of IHNV will continue to increase.

Initially, the host range of IHNV infection and disease outbreaks was thought to be limited to the genus *Oncorhynchus*, but natural infections with epizootics have occurred in other salmonids including *Salmo* spp., *Salvelinus* spp. and *Thymallus* spp. (Table 2.1). Infectious haematopoietic necrosis disease has often been reported in Pacific salmon (sockeye, kokanee, Chinook and chum salmon [*O. keta*]), rainbow trout and steelhead trout (Wolf, 1988) and many other salmon species in Japan (Sano *et al.*, 1977). Cutthroat trout (*O. clarki*) are also susceptible to IHNV infection and disease (Parisot, 1962), but are more resistant than rainbow trout and have been used to generate hybrids to study mechanisms of resistance (Barroso *et al.*, 2008). Coho salmon (*O. kisutch*) and pink salmon (*O. gorbusca*) tend to be less susceptible to disease, but can be infected (Hedrick *et al.*, 1987; Follett *et al.*, 1997). In the genus *Salmo*, Atlantic salmon have had disease outbreaks in fresh water (Mulcahy and Wood, 1986) and very serious epizootics in the marine environment (Traxler *et al.*, 1993, 1997). Atlantic

Table 2.1. Salmonid host range of IHNV.

Common name	Scientific name	Reference
Salmonid species in which natural IHN epizootics have occurred		
Sockeye salmon	*Oncorhynchus nerka*	Rucker *et al.* (1953)
Kokanee salmon	Land-locked *O. nerka*	Sano *et al.* (1977)
Chinook salmon	*O. tshawytscha*	Ross *et al.* (1960)
Chum salmon	*O. keta*	Sano *et al.* (1977)
Cherry salmon (Yamame trout)	*O. masou*	Sano *et al.* (1977)
Biwa salmon	*O. masou rhodurus*	Sano *et al.* (1977)
Amago salmon	*O. rhodurus*	Sano *et al.* (1977)
Rainbow trout	*O. mykiss*	Amend *et al.* (1969)
Steelhead trout	Anadromous *O. mykiss*	Amend *et al.* (1969)
Cutthroat trout	*O. clarki*	Parisot (1962)
Atlantic salmon	*Salmo salar*	Mulcahy and Wood (1986)
Brown trout	*S. trutta*	Yamazaki and Motonishi (1992)
Brook trout	*Salvelinus fontinalis*	Yamazaki and Motonishi (1992)
Japanese char	*S. leucomaenis*	Kimura and Awakura (1977)
Salmonid species of low susceptibility to IHN disease		
Coho salmon	*O. kisutch*	Wingfield and Chan (1970)
Pink salmon	*O. gorbuscha*	Follett *et al.* (1997)
Lake trout	*S. namaycush*	Yamamoto and Clermont (1990)
Arctic char	*S. alpinus*	Follett *et al.* (1997)
Arctic grayling	*Thymallus arcticus*	Follett *et al.* (1997)
Mountain whitefish	*Prosopium williamsoni*	Bootland *et al.* (unpublished)

salmon are considered to be more susceptible to IHNV than sockeye salmon (Traxler *et al.*, 1993). Brown trout (*S. trutta*) are susceptible to natural infections and disease outbreaks (Yamazaki and Motonishi, 1992; Engelking and Kaufman, 1994). *Salvelinus* spp. vary in their susceptibility to IHNV. Lake trout (*Salvelinus namaycush*) can be infected experimentally but appear to be relatively resistant to disease (Yamamoto and Clermont, 1990), and Arctic char (*S. alpinus*) may be completely resistant to IHNV (Follett *et al.*, 1997). Brook trout (*S. fontinalis*) can be infected experimentally (Yamamoto and Clermont, 1990; Goldes and Mead, 1992; Bootland *et al.*, 1994) and have had natural disease outbreaks (Yamazaki and Motonishi, 1992). For other members of the Salmonidae family, Arctic grayling (*Thymallus arcticus*) fry are refractory to infection with a type 1 IHNV isolate and this species is not likely to be a reservoir of IHNV (Follett *et al.*, 1997). Adult mountain whitefish (*Prosopium williamsoni*) can be infected by injection and the virus persists for at least 3 weeks (L.M. Bootland, H.V. Lorz and J.C. Leong, unpublished data). A cell line derived from embryonic tissue of the inconnu or sheefish (*Stenodus leucichthys*) is susceptible to IHNV (Follett and Schmitt, 1990), but it appears that *in vivo* susceptibility tests have not been carried out.

The susceptibility of non-salmonid fish to IHNV has not been studied in depth. Marine species gilt-head sea bream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and sea bass (*Morone labrax*) can be infected experimentally, with resulting mortality in the first two species (Castric and Jeffroy, 1991) and a natural infection reported in an asymptomatic turbot was detected using molecular methods (Liu *et al.*, 2008). It has been demonstrated experimentally that northern pike (*Esox lucius*) fry are highly susceptible to IHNV infection and mortality (Dorson *et al.*, 1987) and susceptibility of pile perch (*Damalichthys vacca*) has been demonstrated experimentally (OIE, 1996). During a wild fish survey in British Columbia, IHNV was detected in one Pacific

herring (*Clupea pallasii*), as well as in tubesnout (*Aulorhynchus flavidus*) and shiner perch (*Cymatogaster aggregata*) collected from a farm experiencing an IHN outbreak (Kent *et al.*, 1998). White sturgeon (*Acipenser transmontanus*) appears relatively resistant to IHNV. Cell lines and larval fish supported IHNV replication, but infection of juveniles or adults was unsuccessful (LaPatra *et al.*, 1995). In Oregon, adult northern squawfish (*Ptychocheilus oregonensis*), adult largescale suckers (*Catostomus columbianus*) and lamprey (*Entosphenus tridentatus*) ammocoetes appear to be refractory to disease and infection after injection of a type 1 IHNV isolate (Bootland, Lorz and Leong, unpublished data). Infectious haematopoietic necrosis virus has been isolated from a few invertebrates: salmon leeches (*Piscicola salmositica*) and ectoparasitic copepods (*Salmincola* spp.) removed from sockeye salmon (Mulcahy *et al.*, 1990), as well as the common mayfly (*Callibaetis* sp.) (Shors and Winston, 1989). The host range of IHNV is likely to continue increasing, but it is important to recognize that there is the potential for non-salmonid fish and invertebrate species to play a role in the life cycle of IHNV by acting as reservoirs or as vectors. Two databases, OIE International Database on Aquatic Animal Diseases (http://www.collabcen.net/toWeb/aq2.asp) and the IHN fish virus database (http://gis.nacse.org/ihnv/index.php) provide valuable resources on IHNV geographic and host range.

Economic Importance of the Disease

IHNV is a major concern in the international salmonid aquaculture industry, as well as government and other agencies in Europe, North America and Japan. Economic losses from IHNV can be substantial in production facilities as a direct consequence of mortality in young fish, where losses can be over 90%. It also threatens propagation programmes and endangered salmon stocks. For example, in 2002, IHN disease in endangered Redfish Lake sockeye in Oregon, USA, resulted in the destruction of 68,000 pre-smolts (Anon., 2002). However, IHNV also causes indirect losses through regulations restricting the movement of IHNV infected fish and management of the disease through diagnostic testing, quarantine, fallowing or destruction of infected fish, resulting in increased overall production costs. From 2005 to 2007, fish with IHNV have been destroyed in Spain, the Czech Republic and Slovenia in attempts to control the spread of IHNV (OIE, 2007). The exact economic cost of IHNV is difficult to determine, but in Alaska in 1992 it was estimated that the total lost revenues from juvenile mortality were US$8.6 million (Meyers *et al.*, 2003). Historically, IHNV was considered as the most important constraint to the profitability and continued growth of the Idaho trout industry, estimated at US$3 million annually (Congleton, 1988). In 2007, the total value of fish sales received by trout growers in the USA totalled US$87.5 million, with Idaho accounting for 53% of the total value of fish sold (US Department of Agriculture, 2008). IHNV has caused up to 50% or higher mortality in young rainbow trout and the presence of spinal deformities in commercial-sized survivors makes these fish less marketable and difficult to process (LaPatra *et al.*, 2001a). As another example of economic importance, IHN epizootics occurred between 2001 and 2003 in over 50% of the Atlantic salmon farms in British Columbia, Canada, resulting in over 12 million salmon dying or being culled and a total direct loss of farm gate revenue of over CAN$250 million (Saksida, 2006; MacKinnon *et al.*, 2008). Clearly, effective methods to control IHN are needed.

Diagnostic Methods

A preliminary diagnosis of IHN can be made based on its previous history and fish showing characteristic clinical signs such as lethargy, dark coloration, distended abdomen, exophthalmia, pale gills and mucoid, opaque faecal casts. Histological observation of necrosis of the granular cells of the alimentary tract is pathognomonic (Wolf,

1988). A preliminary diagnosis must be confirmed by isolation and identification of IHNV. The 'gold standard' for detection of IHNV is the traditional isolation of the virus in cell culture (presumptive diagnosis), followed by specific viral identification using a serum neutralization test. This method is still widely accepted, but other immunological and molecular methods are acceptable for identifying IHNV. Procedures for the isolation and identification of IHNV have been harmonized internationally (OIE, 2006). The Canadian *Fish Health Protection Regulations: Manual of Compliance* (Department of Fisheries and Oceans, 2004) has been revised and there is now a 2007 edition of the American Fisheries Society–Fish Health Section *FHS Blue Book* (AFS–FHS, 2007). Methods to be used for IHNV diagnosis are outlined in detail in each of the above references. Fish tissues to be tested are dependent on fish size and disease status. The preferred tissues are the kidney and spleen; mucus has also been used in non-lethal sampling (LaPatra *et al.*, 1989b). Non-lethal sampling of pectoral fin clips and testing using molecular methods have detected IHNV; hence, this has the potential for a future rapid, high throughput screening system for early detection (Dhar *et al.*, 2008). For testing broodstock, ovarian fluid is preferred, since the virus is detected less frequently in the milt. Sampling of post-spawning females, storage of ovarian fluid or incubation of ovarian fluid cells enhances the sensitivity of viral detection (Mulcahy *et al.*, 1984a; Mulcahy and Pascho, 1986; Mulcahy and Batts, 1987; LaPatra *et al.*, 1990c). Inoculation of ovarian fluid on to suspended EPC cells was more sensitive than the use of EPC monolayers (Hostnik and Jenčič, 2000). If milt is tested for virus, it should be centrifuged and, after the pellet is incubated in water, the water is assayed for the virus (Batts, 1987).

Viral isolation using cell culture

Many teleost cell lines are susceptible to IHNV infection, but samples should be inoculated on to *epithelioma papulosum cyprini* (EPC) and bluegill fry (BF-2) cell lines, as specified in the *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2006). The Canadian *Manual of Compliance* (Fisheries and Oceans Canada, 2004) indicates two of the four recommended continuous cell lines must be used: rainbow trout gonad (RTG-2), Chinook salmon embryo (CHSE-214), EPC or fathead minnow (FHM) cells, with the latter two lines used in IHNV enzootic areas. According to the American Fisheries Society–Fish Health Section (AFS–FHS, 2007) *Blue Book*, only inoculation of EPC cells is required in the USA. EPC cells are the most susceptible to IHNV (Lorenzen *et al.*, 1999). Other fish cell lines susceptible to IHNV cytopathogenicity include steelhead trout embryo (STE-137), CHSE-114, sockeye salmon embryo (SSE-5), SSE-30, kokanee salmon ovary (KO-6), chum salmon heart (CHH-1), rainbow trout hepatoma (RTH-149), guppy embryo (GE-4), coho salmon embryo (CSE-119), rainbow trout spleen (RBS), rainbow trout fry (RTF-1) and Atlantic salmon (AS) cells (Wolf and Mann, 1980; Lannan *et al.*, 1984; Wolf, 1988). The optimum temperature for viral growth is approximately 15°C (Mulcahy *et al.*, 1984b) and 23–25°C does not support viral replication. The virus has also been replicated in baby hamster kidney (BHK/21), reptilian (Clark and Soriano, 1974), *Drosophila melanogaster* (Bussereau, 1975) and *Aedes albopictus* cells (Scott *et al.*, 1980).

Cell culture methods used for detecting and quantifying IHNV are time-consuming and take up to 14 days. End point titrations and plaque assays are typically used, with the latter being more sensitive and considered the 'gold standard' for quantifying IHNV (Fendrick *et al.*, 1982; Batts and Winton, 1989). Factors that affect the plaque assay are inoculum volume, adsorption time and type of overlay (Wolf and Quimby, 1973; Burke and Mulcahy, 1980; Batts and Winton, 1989). Pretreatment of cell monolayers with polyethylene glycol improved the speed and sensitivity of plaque assays and resulted in the production of larger plaques (Batts *et. al.*, 1991). Typical cytopathic effect (CPE) consists of grape-like

clusters of rounded cells, with margination of the chromatin of the nuclear membrane, and typical IHNV plaques consist of a cell sheet that retracts or piles up at the inner margins of the opening and the centre contains granular debris (Wolf and Quimby, 1973). Cells should be examined for 14 days. If no CPE occurs, then the sample is virus-negative, especially if the sample has been passed in tissue culture several times (blind passaged). If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered presumptively positive and confirmatory tests must be completed to identify the virus.

Viral identification

To confirm IHNV, the virus is identified using serological or molecular methods. Serological tests are highly specific and are based on detecting unique viral antigens with antibodies. With advances in molecular techniques, viral identification based on nucleotide sequences has provided increased specificity, sensitivity, accuracy and a faster diagnosis.

Serological methods

Serological assays require either polyclonal or monoclonal antibodies specific to the pathogen. Approved assays for identification of IHNV include serum neutralization, indirect fluorescent antibody test (IFAT), direct alkaline phosphatase immunocytochemistry (APIC) and enzyme-linked immunosorbent assay (ELISA). Other assays available include Western blot, dot blot and staphylococcal coagglutination.

The classic serum neutralization test (SNT) has been optimized and is the serological assay of choice, with methods outlined in numerous regulatory manuals (OIE, 2006; the AFS–FHS *Blue Book* [AFS–FHS, 2007]; Canadian *Manual of Compliance* [Fisheries and Oceans Canada, 2004]). There are at least two variations to the SNT; one uses constant virus concentrations with varying serum dilutions and the other uses a constant serum dilution and varying virus concentrations. The former uses the least amount of serum and is less prone to errors in preparation (Peters and Woodland, 2006). Since IHNV causes plaques, a 50% plaque reduction assay can be used and this is more sensitive than a 50% end point titration (Ahne, 1981). A SNT is not rapid, taking from 7 to 10 days before results are obtained. When coupled with cell culture isolation of the virus, the total time to make a diagnosis can take from 2 to 8 weeks. Obviously, more rapid diagnostic methods had to be developed so that infected fish could be destroyed quickly or quarantined to prevent further spread of the virus.

The IFAT is a rapid, specific and sensitive test that is widely accepted for the detection and identification of IHNV in cell cultures. After cell fixation with formalin or acetone-ethanol, primary unlabelled anti-IHNV antibody (monoclonal anti-IHNV nucleoprotein or glycoprotein, or preadsorbed polyclonal serum) is added, followed by FITC-labelled secondary antibody (LaPatra *et al.*, 1989c; Arnzen *et al.*, 1991; OIE, 2006). A diagnosis can be made in 24 h on cells showing CPE and detects a minimum virus titre of $10^{2.9}$ pfu/ml, but pretreatment of the cells with PEG improves the limit of detection to $10^{2.5}$ pfu/ml. A plaque assay is slightly more sensitive, detecting as low as 10 pfu/ml, but requires up to 8 days to observe plaques and only provides a presumptive diagnosis (LaPatra *et al.*, 1989c). A direct fluorescent antibody test (DFAT) using labelled primary antibody and IFAT have similar sensitivity on cell culture samples, but DFAT can be more rapid (Ristow and Arnzen, 1989; Arnzen *et al.*, 1991). The disadvantages of FATs are that trained personnel are required and fluorescent microscopes are fairly expensive. Direct detection of IHNV in fish tissues using IFAT or DFAT could shorten the time for a diagnosis substantially from several days to a few hours. IFAT detected IHNV in blood smears and kidney imprints from clinically infected fish, but was unsuccessful in detecting virus in smears of seminal fluid (LaPatra *et al.*, 1989c).

Alkaline phosphatase immunocytochemistry (APIC) is an approved method in the *Blue Book* for identifying IHNV infections (AFS–FHS, 2007). This simple and rapid direct assay developed by Drolet *et al.* (1993) can detect IHNV antigen for up to 1 year in formalin fixed and stained tissue culture cells. Virus-infected cell monolayers in plaque assays or 96-well plates are fixed, incubated with primary anti-IHNV monoclonal antibody, followed by biotinylated secondary antibody and avidin-alkaline phosphatase. After adding substrate, the cells are scored for a colour reaction. The test was specific for IHNV, detected all five IHNV electropherotypes and often resulted in a positive reaction prior to visible CPE (Drolet *et al.*, 1993). Horseradish peroxidase or alkaline phosphatase cell-staining techniques have been used frequently in immunohistochemistry to study the pathogenesis of IHNV (Yamamoto *et al.*, 1989, 1990, 1992; Drolet *et al.*, 1994; Kim *et al.*, 1994).

Immunoassays including enzyme-linked immunosorbent assay (ELISA), immunoblots (dot blots) and Western blots all utilize the binding of antigen or antibody to a solid support and detection of virus protein directly with labelled anti-IHNV antibodies or indirectly with labelled secondary antibody. These assays are simple, usually specific and can be quantitative. Virus can be detected once cells begin to show CPE. None of the immunoassays are recommended for detecting IHNV directly from fish tissues.

The ELISA approved by the OIE (2006) for confirming IHNV in cell cultures is a sandwich ELISA based on Dixon and Hill (1984) and Ristow and Arnzen-de Avila (1991). Coating antibody is polyclonal anti-IHNV or MAb against IHNV N protein. IHNV antigen is detected using a biotinylated polyclonal rabbit anti-IHNV antiserum or biotinylated mouse MAb to an N protein epitope different from the one recognized by the coating MAb. This antibody is followed by the addition of streptavidin-conjugated horseradish peroxidase and substrate. The assay requires approximately 22 h to complete. The lowest reported limit of IHNV detection was 10^3 pfu/ml (Ristow and Arnzen-de Avila, 1991), but typically the titre needs to reach 10^6 pfu/ml (Dixon and Hill, 1984).

The immunoblot assay or dot blot is a variation of the ELISA technique and can be used as an additional test to confirm IHNV. Instead of binding antigen to wells, antigen is bound directly to a nitrocellulose or nylon membrane and detected with labelled primary polyclonal or monoclonal antibody, or indirectly with labelled secondary antibody. Immunoblots are faster than ELISA, requiring less than 4 h to complete, and the sensitivity of 10^3–10^6 pfu/ml (McAllister and Schill, 1986; Schultz *et al.*, 1989; Ristow *et al.*, 1991) is comparable to ELISA. Immunoblots have not been successful for identifying IHNV in fish tissue or reproductive samples due to clogging of filters and cross-reacting components (McAllister and Schill, 1986; Schultz *et al.*, 1989).

Western blots are not commonly used in diagnostic laboratories, but are useful for typing and comparing IHNV isolates and determining the specificity of other assays. Virus is grown in cell culture, cell and virus protein are separated by SDS-PAGE and proteins are transferred to a membrane (Kamei *et al.*, 1991). Specific IHNV proteins are detected directly with labelled anti-IHNV antibodies or indirectly with labelled secondary antibody. After cell incubation, the remaining steps require approximately 8 h.

Staphylococcal coagglutination can detect and identify specifically IHNV grown in cell cultures or in infected fish tissue (Bootland and Leong, 1992). This simple test takes only 15 min, making it one of the most rapid methods of diagnosing IHNV. For viruses grown in cell culture, the assay is specific for IHNV, but the test is not very sensitive as 10^6 pfu/ml is required. The test is not suitable for use in cell cultures pretreated with PEG due to non-specific coagglutination. The test has the added benefit of being suitable for field use since it identifies IHNV directly from fry homogenates, adult organs and ovarian fluids when the viral titre is at least 10^6 pfu/g.

Electron microscopy

Electron microscopy and immunoelectron microscopy have been used for research purposes (Helmick *et al.*, 1991; Drolet *et al.*, 1995) but are not typical methods used for diagnosis of IHNV. Visualization of viral particles is very rapid, taking only 3–4 h, and detects 10^2–10^3 particles IHNV/ml of water or 1×10^4 $TCID_{50}$/ml (Leong *et al.*, 1983). If particles have the characteristic bullet shape of rhabdoviruses, a presumptive diagnosis can be made. Immunoelectron microscopy using IHNV-specific antiserum has the advantage of specifically being able to identify IHNV.

Molecular methods

Ideally, diagnostic methods should be suitable for laboratory or field samples and able to detect IHNV carriers that may have very low levels of virus. Molecular methods are the most rapid and sensitive tests currently available and have advanced significantly since the earlier review (Bootland and Leong, 1999). Among the methods used are reverse transcriptase-dependent polymerase chain reaction (RT-PCR), real-time RT-PCR (qRT-PCR), multiplex RT-PCR (mqRT-PCR) and a molecular padlock probe (MPP). With the exception of the latter, an initial RT step must be used to create cDNA from the IHNV RNA genome, followed by amplification by PCR.

RT-PCR has been used frequently to identify IHNV after cell culture passage and is claimed to be more sensitive than traditional virus isolation (Arakawa *et al.*, 1990; Bruchhof *et al.*, 1995; Miller *et al.*, 1998; Barlič-Maganja *et al.*, 2002; Knüsel *et al.*, 2007). RT-PCR offers rapid diagnosis in less than 9 h, but there are some disadvantages, such as the unstable nature of RNA, the risk of contamination and that this method cannot distinguish between infectious and non-infectious virus. RT-PCR, using primers that target the central portion of the *G* gene, is now an approved confirmatory method for IHNV isolated in cell culture (AFS–FHS, 2007). To increase the sensitivity of RT-PCR, the initial amplified product may be reamplified using semi-nested PCR with internal second primers (Miller *et al.*, 1998; Alonso *et al.*, 1999a; Barlič-Maganja *et al.*, 2002). Quantification of the target gene by conventional RT-PCR relies on post-PCR analysis of the amplified product, typically by agarose gel electrophoresis. An alternative is to use digoxigenin-labelled PCR products quantified by an ELISA using specific biotinylated probes (Barlič-Maganja *et al.*, 2002). RT-PCR has been successful when used directly on organ or reproductive products (Miller *et al.*, 1998; Barlič-Maganja *et al.*, 2002; Knüsel *et al.*, 2007) but is not yet approved for diagnosis by regulatory authorities. In the case of an acute outbreak of disease where a rapid result is required, the shorter processing time and higher sensitivity makes RT-PCR the method of choice for confirmation of the suspected disease (Knüsel *et al.*, 2007).

Real-time RT-PCR (qRT-PCR) has a greater sensitivity than conventional RT-PCR, requires little initial RNA, has a wide dynamic detection range, does not require post-PCR analysis and can be formatted for high throughput applications (Dhar *et al.*, 2008). This technique is well suited for diagnostic testing and for estimating viral load as it confirms and quantifies IHNV easily in samples such as cells, tissues, water or food (Overturf *et al.*, 2001). Different methods have been employed to detect amplicons generated by qRT-PCR. Initially, fluorescent-labelled probes to the *N* and *G* gene used in a TaqMan assay detected IHNV successfully in the kidney and brain of IHNV infected fish (100 copies/reaction), showing that this assay would be valuable for detecting IHNV carriers or reservoirs (Overturf *et al.*, 2001). A similar assay using dual-labelled fluorescent probes to the *G* gene could quantitate genomic RNA (negative-strand specific) or mRNAs and antigenome molecules (positive-strand specific), which would indicate if the virus was actively replicating (Purcell *et al.*, 2006a). Although not capable of distinguishing between live and dead virus, qRT-PCR using negative sense primers was more sensitive for detecting IHNV in rainbow trout liver than plaque assays (Purcell *et al.*, 2006a). Multiplex qRT-PCR (mqRT-PCR) would

allow detection of more than one pathogen within the same sample. A single tube mqRT-PCR assay using fluorescent-labelled TaqMan probes for the simultaneous detection of three fish rhabdoviruses (spring viraemia of carp virus, IHNV and viral haemorrhagic septicaemia virus) was first used by Liu *et al.* (2008) and was found to be as sensitive as cell culture in detecting IHNV in naturally infected asymptomatic turbot in China. A less expensive qRT-PCR assay using the DNA-binding fluorophore SYBER green I instead of labelled probes detected IHNV *N* and *G* genes successfully in infected cell cultures and several rainbow trout tissues, including the pectoral fin (Dhar *et al.*, 2008). This assay was as sensitive as cell culture in detecting IHNV in tissues from rainbow trout infected in the laboratory or healthy fish collected from commercial operations, but was less effective in detecting IHNV in naturally infected fish showing clinical signs (Dhar *et al.*, 2008). Potential reasons cited for this discrepancy were the presence of more than one viral strain in the field samples and the inability to detect all the strains equally by qRT-PCR with the primer sets used in the study.

Molecular padlock probe (MPP) technology, first used for IHNV by Millard *et al.* (2006), is an alternative molecular detection strategy that uses a single-stranded linear probe oligonucleotide containing bases at the 5′ and 3′ ends that are compatible with a shorter, single-stranded target sequence. MPPs are employed in an isothermal, non-PCR-based nucleic acid amplification system that yields a circular oligonucleotide construct that then undergoes rolling circle amplification and can be further amplified by hyperbranching techniques. MPP was successful in detecting 10^4 DNA oligonucleotide targets and detected IHNV in 50 μg total RNA from the kidney tissue of infected rainbow trout, comparable to the sensitivity of RT-PCR (Millard *et al.*, 2006).

Although molecular methods have been shown to be highly specific, sensitive and accurate, and can be used to achieve rapid identification of IHNV in field and laboratory samples, there is a need to validate and standardize protocols and interpretation before being approved for the detection, identification and quantification of IHNV.

Detection of anti-IHNV antibodies in fish serum

Salmonids infected with IHNV may mount a strong antibody response that persists for months after infection (Hattenberger-Baudouy *et al.*, 1995). Monitoring the antibody response does not require lethal sampling and may be useful in determining previous IHNV exposure and in IHNV surveillance programmes (LaPatra, 1996; St-Hilaire *et al.*, 2001). However, due to variations in the strength and duration of the serological response and lack of validated and standardized procedures, detection of fish antibodies has not yet been accepted as a routine diagnostic method for assessing the viral status of fish populations (OIE, 2006). Fish may have elevated antibody titres but test negative for IHNV infection (Traxler *et al.*, 1997; St-Hilaire *et al.*, 2001) and if this occurs, it remains uncertain which control measures are needed (Knüsel *et al.*, 2007). Conventional assays to monitor antibody levels are the serum neutralization test and plaque reduction assay, which both require a complement source, but IFAT or ELISA have also been used (Hattenberger-Baudouy *et al.*, 1989; Jørgensen *et al.*, 1991). Ristow *et al.* (1993) suggested that ELISA could replace the plaque neutralization test for detecting anti-IHNV antibodies because the two assays were of similar sensitivity, but ELISA required less time to complete. ELISA is valuable for screening large numbers of samples, is relatively specific, reproducible and is of low cost in time and materials.

Genome Structure and Transcription

Viral genome

The complete genome sequence of IHNV has been reported by Morzunov *et al.* (1995) for a Western Regional Aquaculture Consortium (WRAC) IHNV isolate and by Schutze *et al.*

(1995) on a virus isolate from Pierre de Kinkelin in 1987. This paper indicated that the 'original isolate was obtained from Oregon in rainbow trout in 1969'. The viral genome is 11,131 nucleotides (nt) in length and contains a 60 nt leader sequence at its 3′ end and a 101 nt trailer sequence at its 5′ end. The 3′ and 5′ ends of the IHNV genome are complementary, just like the ends of the vesicular stomatitis virus (VSV) and the rabies virus (RV) genomes, and are thought to provide the means for the viral RNA to form panhandle structures for priming the initiation of RNA synthesis (Banerjee and Barik, 1992). The gene order from the 3′ end of the genome is 3′ leader-*N*-*P*-*M*-*G*-*NV*-*L*-trailer-5′.

The internal gene junctions of IHNV are different from those of other rhabdoviruses. Typically, the gene junctions for the VSV and RV contain the sequence, $UC(U)_7NNUUGU$. In contrast, the consensus intergenic region for the fish rhabdoviruses is $UC(U)_7RCCGUG$, where R is a T or C. Thus, during mRNA synthesis, the polymerase, which initiates transcription at the AACA sequence for both VSV and RV, initiates transcription at T/CGGCAC for IHNV (P.P. Chiou, unpublished data).

Messenger ribonucleic acid transcription

Six viral mRNA species have been identified in cells infected with IHNV (Kurath and Leong, 1985) and each of these mRNA species has been cloned and sequenced for a number of IHNV isolates (Leong and Kurath, 2010). The N mRNA is presumably the first mRNA species produced during viral replication, since N is the first viral protein that appears in infected cells. When the relative concentration of each mRNA species is measured at the height of viral replication and the N mRNA concentration is set at 1, the relative concentration of P and M mRNAs are 2.52±0.40; G mRNA is 0.49± 0.03; NV mRNA is 0.41±0.14; and L mRNA is 0.02±0.01 (Kurath and Leong, 1985).

The mRNAs of rhabdoviruses are considered to be monocistronic, although read-through transcription products have been identified for VSV (Masters and Samuel, 1984), RV (Ravkov *et al.*, 1995) and the ephemeroviruses (Wang *et al.*, 1995). No read-through transcripts have been identified for IHNV (P.P. Chiou, unpublished data). However, there is a possibility that a single IHNV mRNA species might encode more than one translation product. Since there are many examples of different translation products either encoded in different reading frames, initiated at a different start codon or resulting from an internal frame shift that leads to a hybrid protein product, the possibility that there are additional proteins encoded by the IHNV genome is very high. For example, the paramyxoviruses and rhabdoviruses have encoded in their P mRNA transcripts for more than one protein (Lamb *et al.*, 1976; Curran *et al.*, 1992; Spiropoulou and Nichol, 1993). In fact, a seventh protein, S for small protein, at 6.5 kDa, has been described in IHNV-infected cells (Chiou, 1996). Whether this S protein is encoded by one of the IHNV mRNA transcripts has not been determined.

An *in vitro* transcription system for purified virions of IHNV was first described by McAllister and Wagner in 1977. They described a heterogeneous array of IHNV-specific transcripts, ranging in size from 9S to 17S, with no discrete species identified. Later studies by Kurath and Leong (1987) demonstrated that optimal polymerase activity required HEPES buffer supplemented with *S*-adenosyl-L-methionine. In this case, RNA transcripts contained polyadenylated species, which co-migrated with the IHNV N, P, M, G and NV mRNAs from IHNV infected cells. These transcripts were fully functional in translation reactions with rabbit reticulocyte extracts to produce N, P and M proteins.

The process by which IHNV replicates its viral genome is presumed to be similar to that described for the vesiculoviruses and lyssaviruses (Banerjee, 1987). In this case, the viral N protein provides the trigger that switches the RNA transcription process from mRNA synthesis to progeny genome (+) synthesis. The viral N protein is present in such large quantities in the late phase of viral replication that it binds to the nascent RNA

transcript and prevents the viral polymerase from recognizing the mRNA transcription termination signals. This results in the synthesis of a full-length plus strand template for the production of progeny minus strand viral genomes. Studies by Biacchesi *et al.* (2000a,b) using reverse genetics to synthesize recombinant IHNV virus from T7-generated minigenomes suggest that the mechanisms involved in viral replication are similar.

Viral gene expression

Infection of salmon or trout cells with IHNV results in the inhibition of cellular protein synthesis. In this characteristic, IHNV differs from other members of the RV group of the Rhabdoviridae. Cellular protein synthesis is not inhibited after infection with RV (Coslett *et al.*, 1980) and any studies of RV protein synthesis in the cell require exposure to hypertonic shock to reduce the background of host protein synthesis. Thus, it is possible to examine the synthesis of IHNV proteins in the cell without resorting to this drastic treatment. In pulse-labelling studies with ^{35}S-methionine, the first protein of IHNV to appear in the course of infection is the N, or nucleocapsid, protein, at 2–3 h after infection (Hsu *et al.*, 1985). At 6–7 h after infection, the P and M proteins can be identified in autoradiograms. The two forms of the glycoprotein, G1 and G2, representing different glycosylation states of the protein, are found at 9–10 h after infection. It is not possible to distinguish the virion L protein from other host proteins in the gel until 15 h post-infection, when cellular host protein synthesis is completely inhibited. Virus production begins 12 h after infection (Leong *et al.*, 1981).

Viral proteins

Nucleocapsid protein

The N protein is the most abundant viral protein in the IHN virion and in virus-infected cells. It is present as a phosphorylated protein in the virion (McAllister and Wagner, 1975; Hsu *et al.*, 1985; Koener *et al.*, 1988) and is found tightly associated with the viral genome (Engelking and Leong, 1989a,b). The complete nucleotide sequence of the *N* gene has been determined for numerous isolates of IHNV and these data contribute to the phylogeography of the virus in North America (Kurath *et al.*, 2003) and this can be used to trace the origin of the virus in disease outbreaks. The *N* gene encodes a protein of 413 amino acids, which is highly hydrophilic at its amino and carboxyl terminal ends, with a hydrophobic region in the middle third of the protein. It has been speculated that the carboxyl terminal end of the IHNV N protein may be involved in binding of the N protein to the viral RNA (Gilmore and Leong, 1988). For the prototype rhabdovirus VSV, the N protein has been shown to play a crucial role in regulating the balance between viral transcription and replication.

Phosphoprotein

The P protein, previously called M1, is a phosphorylated protein of 230 amino acids with a calculated molecular weight of 25.6 kDa (McAllister and Wagner, 1975; Hsu *et al.*, 1985; Ormonde, 1995). Sequence analysis of the gene encoding the P protein for IHNV RB-1 and KS-1 strains indicate that the deduced amino acid sequence contains 45 potential phosphorylation sites, SXXD/E, which are found predominantly in the first half of the protein (Ormonde, 1995). The P protein is a very basic protein with an estimated isoelectric point (pI) of 8.4, similar to that reported for the VHSV (Makah strain) P protein (Benmansour *et al.*, 1994; Ormonde, 1995). The basic nature of these proteins contrasts sharply with the RV and VSV phosphoproteins that have pIs of 4.36 and 4.84, respectively. The P proteins of the RB-1 strain and the European KS-1 strain of IHNV share a high degree of similarity, with 94% identity at the amino acid level (Ormonde, 1995; Morzunov *et al.*, 1995). When compared with the P proteins for HIRRV and VHSV, a 63% and 38% similarity was observed between these proteins and

the IHNV M1 protein (Ormonde, 1995). Sequence analysis of the *P* genes of different IHNV strains indicates that there might be a second overlapping open reading frame (ORF) encoding a highly basic, arginine-rich protein, with an estimated pI of 10.1–12.8. Located at position 121 from the end of the polyadenylated sequence of the *N* gene, this second ORF is 146 nucleotides in length and has the potential to encode a 42 amino acid protein with an estimated molecular weight of 4.8 kDa. The protein is similar in size to the 55 amino acid, arginine-rich C protein reported for VSV (Spiropoulou and Nichol, 1993) and RV (Chenik *et al.*, 1995). The VSV C protein is also encoded by the P mRNA transcript in a second overlapping ORF within the gene. These proteins are found only in infected cells and their function is currently not known.

Matrix protein

The IHNV matrix protein (M), previously called M2, is similar to all reported rhabdovirus matrix proteins (Ormonde, 1995). The M protein is encoded by a sequence of 585 nucleotides to yield a protein of 195 amino acids, with 21.8 kDa estimated molecular weight. It is very basic, with an estimated pI of 10.08, similar to the M proteins of the other rhabdoviruses, and contains a high concentration of charged amino acids in the amino terminal half of the protein. Highly charged amino termini have also been found in the vesiculoviruses and the lyssaviruses (Rose and Gallione, 1981; Bourhy *et al.*, 1993) and in paramyxoviruses (Chambers *et al.*, 1986). In VSV, the first 51 amino acids are required for stable interaction with the plasma membrane (Chong and Rose, 1994), for viral assembly (Black *et al.*, 1993) and possibly in the inhibition of RNA transcription (Ogden *et al.*, 1986). This region is also conserved in IHNV, HIRRV and VHSV matrix proteins.

The deduced IHNV M amino acid sequence does not share significant homology with VSV, RV or the fish vesiculovirus, spring viraemia of carp virus (SVCV). However, it shares a 74% amino acid identity with HIRRV M protein and a 37% identity with the VHSV M protein at three localized regions of homology (Ormonde, 1995). Sequence conservation is highest in the N-terminal region of the first 29 amino acids that contains a large number of basic residues. This feature has also been described for the amino termini of RV and VSV.

The IHNV M protein does induce apoptosis in cells transfected with a DNA plasmid, pM(+), which drives the expression of the M protein through the cytomegalovirus immediate early gene promoter (Chiou *et al.*, 2000). Typical fragmented nuclei and reduced expression of cellular RNA, as well as reduced cellular protein expression, were observed by immunofluorescence confocal microscopy. The cellular pathway used by the IHNV M protein to induce apoptosis has not been identified. Wild-type VSV has been known to induce apoptosis by activating the caspase cascade at caspase 9 (Kopecky and Lyles, 2003).

Glycoprotein

The IHNV glycoprotein G is a membrane-associated protein that forms spike-like projections on the surface of the mature virion (McAllister and Wagner, 1975). Antiglycoprotein serum neutralizes viral infectivity, and immunization with purified glycoprotein prevents subsequent lethal infection with IHNV (Engelking and Leong, 1989b). Immunological studies with polyvalent antiglycoprotein sera have indicated that the glycoproteins are conserved among different geographical isolates of IHNV (Hsu and Leong, 1985) and that there is only one major serotype with several serovariants (Engelking and Leong, 1989a). This finding has been confirmed with MAb (Huang *et al.*, 1996) and sequence analysis of the *G* and *NV* genes (Nichol *et al.*, 1995).

The IHNV glycoprotein has many of the features characteristic of membrane-associated glycoproteins of negative-stranded RNA viruses. The predicted translation product of the IHNV *G* gene is a protein of 508 amino acids, with a hydrophobic domain of 20 amino acids at the N terminus forming the signal peptide. This includes a central core of hydrophobic amino acid residues, in

the form of three repeated pairs of Leu–Ile (Koener *et al.*, 1987) and a consensus signal peptide cleavage site, Ala–Asn–Ser, at position 18. The arrangement of the amino acids in this region is consistent with the signal peptidase cleavage sequences identified by Perlman and Halvorson (1983). There is an additional hydrophobic domain near the C terminus, which is the presumed transmembrane domain of the protein.

A comparison of the predicted amino acid sequences of the glycoproteins of the vertebrate rhabdoviruses suggests that these proteins are highly similar in structure. The cysteine residues of the IHNV G protein are highly conserved amino acids, whose positions in the G proteins of VSV-New Jersey (VSV-NJ), VSV-Indiana (VSV-Ind) and RV are largely identical. In the case of VSV-Ind, 12 of 15 cysteine residues are aligned with IHNV at 12 of its 16 cysteine residues. For RV, nine are aligned with the 17 cysteine residues in the IHNV G protein. When the positions of the proline residues are aligned, approximately one-third of the proline positions between RV and IHNV, as well as between VSV and IHNV, are identical. However, a hydropathy plot of the G proteins did reveal a striking difference between them. The profiles revealed a very hydrophilic domain extending from IHNV G amino acid 365 to 450, which was not evident in either VSV or RV. Within this region were two possible glycosylation sites and no cysteine residues. The importance of this region as a possible antigenic domain was first suggested by Koener *et al.* (1987). A more recent study by Bjorklund *et al.* (1996) expanded the comparison of the fish rhabdovirus glycoproteins to the glycoproteins of other rhabdoviruses. The analysis found that the amino acids cysteine, proline, glycine and leucine were conserved in the aligned glycoprotein sequences.

Non-virion gene

The *NV* gene and its encoded protein were first identified in 1985 (Kurath and Leong, 1985; Kurath *et al.*, 1985). Located between the *G* and *L* genes, *NV* is 371 nt in length and encodes a protein of 111 amino acids. The overall pI of the deduced protein is approximately 7, despite the fact that the protein has a high content of charged amino acids (38%) (Nichol *et al.*, 1995). An NV protein has also been identified at the G–L junctions of HIRRV and VHSV, a finding that has prompted scientists to propose a new taxonomic classification for the salmonid fish rhabdoviruses (Schutze *et al.*, 1996; Kurath *et al.*, 1997). The gene is highly conserved. Among 14 different IHNV isolates, comprising 13 isolates from North America and one from Europe, more than 97% identity at the amino acid level was observed (Nichol *et al.*, 1995; Schutze *et al.*, 1995; Chiou, 1996; Kurath *et al.*, 1997). The NV amino acid identity/similarity values between IHNV and VHSV or HIRRV indicate that this protein is highly conserved, with identity/similarity values of 23.3/47.6% and 16.5/40.4%, respectively (Kurath *et al.*, 1997). There are sufficient differences in the *NV* genes among the different IHNV isolates for ribonuclease (RNAse) protection assays to have been used to distinguish the isolates (Kurath *et al.*, 1995).

Characterizing the expression of the NV protein in infected cells has been problematic. In the original report by Kurath and Leong (1985), the NV protein was clearly observed in autoradiograms of SDS-PAGE gels containing lysates of infected cells labelled with ^{35}S-methionine or the *in vitro* translation products of mRNA from infected cells. Subsequent reports have shown that the expression of NV is very low or below detection limits for IHNV using radiolabelling (Chiou, 1996), VHSV (Basurco and Benmansour, 1995) and HIRRV (Nishizawa *et al.*, 1991a,b). More recently, NV protein expression was detected in cells infected with either IHNV or VHSV using immunofluorescence and Western immunoblot (Schutze *et al.*, 1996).

Although there has been much speculation concerning the function of NV (Kurath and Leong, 1985; Nichol *et al.*, 1995; Chiou, 1996), the role NV plays in the replication of the virus remains unknown. No functional motifs have been identified in the protein sequence and no significant homology to

other protein sequences in the GenBank database has been uncovered (Basurco and Benmansour, 1995; Nichol *et al.*, 1995). NV in Novirhabdoviruses has been found to be essential in IHNV replication (Thoulouze *et al.*, 2004) and non-essential in the replication of the warmwater snakehead rhabdovirus (Johnson *et al.*, 2000; Alonso *et al.*, 2004). The difference in findings is dramatic and it is not clear whether it is due to host cell-line characteristics and/or temperature.

Polymerase

The *L* gene (nt 5016 to nt 10,976) of IHNV encodes a protein of 1986 amino acids with an estimated molecular mass of 225.2 kDa (Bjorklund *et al.*, 1995; Morzunov *et al.*, 1995; Schutze *et al.*, 1995). The IHNV polymerase is similar to the VSV polymerase, which has been shown to be solely responsible for all transcriptional activities. Thus, the IHNV polymerase should also be capable of RNA-dependent RNA polymerization, cap methylation and poly-A polymerization. The deduced amino acid sequence contains the six conserved blocks of amino acids identified as conserved sequences in negative stranded RNA polymerases (Poch *et al.*, 1990). An examination of the phylogenetic relationships among the rhabdoviruses, inferred by using the polymerase gene, indicates that the Novirhabdoviruses cluster more closely with the plant rhabdoviruses, Nucleorhabdovirus and Cytorhabdovirus. The rhabdoviruses of terrestrial animals are more closely related to each other (Bourhy *et al.*, 2005).

Pathogenesis and Immunity

Disease progression

The routes of viral entry after waterborne challenges are through the gill, skin, fin bases, oral region and the oesophagus/cardiac stomach region, with viral replication initially occurring in epidermal cells (Mulcahy *et al.*, 1983b; Yamamoto *et al.*, 1990, 1992; Helmick *et al.*, 1995a,b; Arkush *et al.*, 2004; Foott *et al.*, 2006; Harmache *et al.*, 2006). The fin bases, gills and skin of rainbow trout are viewed as initial and transient sites of viral replication before the virus spreads to the internal organs (Yamamoto and Clermont, 1990; Drolet *et al.*, 1994; Cain *et al.*, 1996; Brudeseth *et al.*, 2002; Harmache *et al.*, 2006). In contrast, the gill and skin of Chinook salmon juveniles may remain infected for a longer time (39 days) and appear to support viral growth in the absence of an extended involvement of internal organs (Foott *et al.*, 2006).

The haematopoietic tissues of the kidney and spleen of young fish are the most severely affected and are the first tissues to show extensive necrosis (Amend *et al.*, 1969; Yasutake, 1970, 1975). Typically, within a day after exposure, low titres of IHNV are detectable in gills, skin and intestine of young rainbow trout before the infection spreads to the kidney (2–4 days) and subsequently becomes widespread throughout the organs (Yamamoto and Clermont, 1990; Drolet *et al.*, 1994; Brudeseth *et al.*, 2002). Viral prevalence and titres peaked within 2 weeks of infection and then began to decrease. Fish were still infected at 28 days, but the virus was no longer detectable after 54 days (Drolet *et al.*, 1994). Drolet *et al.* (1994) proposed that viral infection progressed by two major routes: from the gill into the circulatory system and from the oral region into the gastrointestinal (GI) tract and then the circulatory system, with both routes resulting in systemic viraemia. They also proposed that the GI route was not responsible for initial infection of the kidney based on finding that ingested virus transiently infected epithelial cells lining the oral cavity and GI tract, then spread to basally adjacent, highly vascularized connective tissue (submucosa and/or lamina propria) of all GI organs, which resulted in infection of the heart and systemic dissemination. IHNV infected epithelial cells readily but transiently, but had a propensity for connective tissue in numerous organs. Brudeseth *et al.* (2002) indicated that the skin did not appear to be a portal of virus entry and found that, in kidney, endothelial cells lining the sinusoids were infected,

then the virus spread from the reticuloendothelial stroma to the interstitial leucocytes and melanomacrophages. In the spleen, the virus was localized in ellipsoidal macrophages. An ultrastructural study confirmed that an early IHNV target area was the oesophagus/cardiac stomach region (ECSR), particularly the cardiac mucus secreting cardiac gland (MSSG) (Helmick *et al.*, 1995a,b). There was evidence of attachment and internalization of IHNV in the ECSR mucosal epithelial cells in rainbow trout and coho salmon within 1 h post-infection. The MSSG of coho salmon had a milder reaction, indicating that the virus replicated less efficiently in coho salmon. In Chinook and sockeye salmon fry, there might be hepatic deposits of ceroid (Wood and Yasutake, 1956; Yasutake, 1970). In the final stages of disease, necrosis was not only in the haematopoietic tissue of the kidney, but also in the glomeruli and kidney tubules. There was little effect on the corpuscles of Stannius tissue (Yasutake, 1975; Wolf, 1988). IHNV survivors could have spinal curvature deformities including scoliosis and lordosis, but rainbow trout survivors reared to commercial size had a low prevalence of spinal deformities (LaPatra *et al.*, 2001a).

Sexually mature salmonids from several species have natural infection rates varying from 0 to 100% (Mulcahy *et al.*, 1983b; Follett *et al.*, 1987; LaPatra *et al.*, 1989a; Meyers *et al.*, 1990, 2003). The gills are infected most frequently in pre-spawning sockeye salmon, followed in descending order by the spleen, lower gut, kidney, eggs, pyloric caeca, liver and brain, and with no virus in the serum (Mulcahy *et al.*, 1982). The prevalence of infection and viral titre tends to increase from pre-spawning to spawning to post-spawning in sockeye salmon females, but results vary from year to year and not all fish are infected (Meyers *et al.*, 1990, 2003). This is logical since post-spawned fish have an increased time for viral exposure because they have been on the spawning grounds longer and are more physiologically spent than fish that have arrived more recently (Meyers *et al.*, 2003). In spawning and post-spawning sockeye salmon and Chinook salmon females, gills are thought to be the virus entry point, with high titres also in the spleen and ovarian fluid, although the virus is found in all organs and the serum (Mulcahy *et al.*, 1982, 1983b; Yamamoto *et al.*, 1989; Arkush *et al.*, 2004). Female sockeye salmon show a higher prevalence of infection than males, which may be due to hormone levels or difficulties in detecting IHNV in milt (Mulcahy *et al.*, 1983b, 1984b; LaPatra *et al.*, 1989a; Yamamoto *et al.*, 1989; Meyers *et al.*, 1990). Water elution of virus from sperm enhances detection (Batts, 1987), but many studies do not appear to have used this method.

Histopathological findings reveal degenerative necrosis in haematopoietic tissues, posterior kidney, spleen, liver, pancreas and digestive tract. In the anterior kidney, initial changes are small, lightly stained focal areas consisting of what appear to be macrophages and degenerating lymphoid cells. As the disease progresses, degenerative changes throughout the kidney become more noticeable. Macrophages increase in number and may have a vacuolated cytoplasm and chromatin margination of the nuclei, and pyknotic and necrotic lymphoid cells may be present (Klontz *et al.*, 1965; Yasutake and Amend, 1972). Necrosis may be so severe that the kidney tissue consists primarily of necrotic debris (Yasutake, 1975; Wolf, 1988). Focal areas of cells in the spleen, pancreas, liver, adrenal cortex and intestine show nuclear polymorphism and margination of the chromatin, with eventual necrosis (Amend *et al.*, 1969; Yasutake, 1975; Wolf, 1988). Extensive necrosis in all organs is accompanied by pyknosis, karyorrhexis and karyolysis (Yasutake and Amend, 1972). A pathognomonic feature of IHN is degeneration and necrosis of granular cells in the lamina propria, stratum compactum and stratum granulosum of the alimentary tract (Yasutake, 1970; Wolf, 1988), and it is postulated that sloughing of intestinal mucosa may give rise to faecal casts (Amend *et al.*, 1969; Yasutake, 1970, 1975). Smolts and yearlings tend to show less severe histopathological changes. The kidney, spleen, pancreas and liver may show necrosis, but there is only moderate sloughing of the

intestinal mucosa and no faecal casts (Yasutake, 1978; Burke and Grischkowsky, 1984; Traxler, 1986; Yamamoto *et al.*, 1989). Fish have a normocytic aplastic anaemia and the blood of affected fish shows leucopenia with degenerating leucocytes and thrombocytes, reduced haematocrit and osmolarity and a slightly altered biochemical profile (Wood and Yasutake, 1956; Holway and Smith, 1973; Amend and Smith, 1974, 1975). Cellular debris, termed necrobiotic bodies, observed in blood smears or kidney imprints, is pathognomonic for IHN (Wolf, 1988).

Predisposing factors

Host factors

The ability of IHNV to cause disease and mortality is dependent on host factors such as fish species, age and weight (LaPatra *et al.*, 1990a,b, 1993a; Basurco *et al.*, 1993), stock (Wertheimer and Winton, 1982) and family group (Amend and Nelson, 1977; Mclntyre and Amend, 1978; Kasai *et al.*, 1993; Rodriguez *et al.*, 2004; Quillet *et al.*, 2007). IHN typically causes disease in salmonid fish, but susceptibility varies with species, as discussed earlier (section on host range). Fish typically become more resistant to IHNV as they increase in age and weight (Amend and Nelson, 1977; LaPatra *et al.*, 1994; Troyer *et al.*, 2000; Bergmann *et al.*, 2003). Yolk-sac fry and fish up to 2 months of age are highly susceptible, with mortality often over 90% (Traxler and Rankin, 1989). Two- to 6-month-old fish usually have less than 50% mortality. IHN can kill older sockeye salmon (Yasutake, 1978; Burke and Grischkowsky, 1984), kokanee salmon (Traxler, 1986) and rainbow trout (Busch 1983; Kim *et al.*, 2007), but mortality is generally low. However, high mortalities (>45%) have occurred in marine farmed Atlantic salmon, with smolts being most susceptible and mortality decreasing with increasing fish size and age (Armstrong *et al.*, 1993; St-Hilaire *et al.*, 2002; Saksida, 2006). The host species from which the IHNV isolate is obtained has a negligible effect on pathogenicity for other salmonids (LaPatra *et al.*, 1989a, 1990a; Chen *et al.*, 1990; Traxler *et al.*, 1993).

Viral factors

Viral titres in excess of 10^4 pfu/g of tissue are usually found in infected fish (Mulcahy *et al.*, 1983b; Burke and Grischkowsky, 1984; Traxler, 1986). Viral dose (10^2–10^6 pfu/ml) is correlated directly with mortality in several species of fry and juvenile fish (Engelking and Leong, 1989b; LaPatra *et al.*, 1989a, 1990a, 1993a; Bootland *et al.*, 1994; Follett *et al.*, 1997; Foott *et al.*, 2006). An IHNV challenge protocol for any fish species or size has not been standardized between laboratories, or approved. As a result, variations in experimental parameters such as viral isolates, viral doses and routes to infect fish of different species and sizes have been used by investigators, making it difficult to draw conclusions on isolate virulence.

It is clear that numerous strains of IHNV exist and phenotypic and genetic relatedness generally correlates with geographic origin and that different fish species within an area harbour the same type of IHNV (Hsu *et al.*, 1986; Winton *et al.*, 1988; Nichol *et al.*, 1995; Oshima *et al.*, 1995; Kurath *et al.*, 2003). Although isolate virulence with induction of mortality appears to show some host specificity, virulence of isolates is variable and cannot be predicted reliably. Based on electropherotypes (Hsu *et al.*, 1986), type 1 IHNV isolates are more virulent in kokanee, sockeye salmon and brook trout and type 2 isolates are more virulent in rainbow trout and steelhead trout (Amend and Nelson, 1977; LaPatra *et al.*, 1990a,b, 1993a; Bootland *et al.*, 1994). Of the three genogroups based on the *G* gene occurring in North America, the U (upper) genogroup primarily contains isolates from sockeye salmon, with several isolates from Chinook salmon and steelhead trout (Emmenegger *et al.*, 2000; Emmenegger and Kurath, 2002; Garver *et al.*, 2003; Kurath *et al.*, 2003). Saltwater challenge of adult salmonids with a U type isolate from sockeye salmon showed a higher virulence in Atlantic salmon than in

sockeye salmon, with Chinook salmon being resistant (Traxler *et al.*, 1993). In Japan, isolates belonging to the U genotype have caused mortality in numerous salmonid species, but a new genotype, JRt (Japanese rainbow trout), affecting rainbow trout has been identified (Nishizawa *et al.*, 2006). In Russia, only the U genotype has been isolated and, although wild and broodstock sockeye salmon appear to be the main host, epidemics in rainbow trout fry have occurred (Rudakova *et al.*, 2007). The M (middle) genogroup is found principally in rainbow trout in the USA (Troyer *et al.*, 2000; Garver *et al.*, 2003) and Western European countries (Enzmann *et al.*, 2005; Kolodziejek *et al.*, 2008) and appears to be avirulent in sockeye salmon (Batts *et al.*, 2005). The L (lower) genogroup is found principally in Chinook salmon, although there are also several steelhead isolates (Kurath *et al.*, 2003). Chinook salmon appear to be more susceptible to electropherotype 3 isolates (L genogroup); however, virulence of isolates within the same electropherotype is variable and is affected by the species being challenged (Chen *et al.*, 1990; LaPatra *et al.*, 1993a; Bendorf *et al.*, 2007).

Numerous IHNV isolates have been partially sequenced and sequence analysis of regions within the glycoprotein gene revealed a maximum nucleotide diversity of 8.6%, indicating low genetic diversity for this virus (Troyer *et al.*, 2000; Garver *et al.*, 2003; Kurath *et al.*, 2003; Enzmann *et al.*, 2005; Nishizawa *et al.*, 2006; Kolodziejek *et al.*, 2008). Based on the low genetic diversity in the *G* gene, LaPatra *et al.* (1993a) suggested that the glycoprotein was not responsible for virulence. However, amino acid changes within the *G* gene were detected and this potentially could result in altered secondary structure with changes in tissue tropism and virulence (Kim *et al.*, 1994; Huang *et al.*, 1996; Troyer *et al.*, 2000; LaPatra *et al.*, 2008). In Idaho, four virus lineages were identified based on phylogenetic analyses and found to be circulating within and between facilities (Troyer *et al.*, 2000). Differences in isolate virulence were observed even within the same virus lineage, but the high and potentially increasing diversity of IHNV in trout farms did not appear to be associated with a change in virulence over the two periods examined (Troyer *et al.*, 2000). Several hot spots for amino acid substitutions in the G protein of Idaho isolates have been noted (Troyer *et al.*, 2000; LaPatra *et al.*, 2008), but it is unknown if these changes are related to changes in virus virulence. It is possible that changes in other IHNV genes may affect virulence. For example, the *NV* gene is considered as a virulence factor and has been shown to be essential for IHNV pathogenicity in rainbow trout (Thoulouze *et al.*, 2004). In addition to detecting antigenic differences based on the G protein, monoclonal antibodies have detected heterogeneity in the nucleoprotein (Ristow and Arnzen, 1989; Ristow and Arnzen-de Avila, 1991). The relation between virulence and heterogeneity in the nucleoprotein has not been determined.

Another factor that can affect IHNV virulence is a co-infection with another virus. Experimental IHNV and IPNV co-infections of BF2 cells (Alonso *et al.*, 1999b) or rainbow trout fry (Alonso *et al.*, 2003a; Byrne *et al.*, 2008) resulted in lower IHNV replication and significantly lower fish mortality compared with a single infection with either virus alone. For VHSV and IHNV co-infections, both viruses establish an infection in rainbow trout with similar viral titres in the kidney compared with a single infection, but co-infection results in a more restricted IHNV organ distribution due to some degree of interaction at the cellular level (Brudeseth *et al.*, 2002). The mechanism through which IPNV interferes with IHNV replication appears to be at the viral binding step, suggesting competition for viral receptors (De las Heras *et al.*, 2008) and, although not proven, a similar situation may be occurring for VHSV and IHNV infections.

Environmental and management factors

The most important environmental factor affecting IHNV virulence is temperature. Natural IHN epizootics occur most frequently during the spring and autumn in

the northern hemisphere when water temperatures are 8–14°C and typically do not occur above 15°C (Nicholson, 1982). Elevation of water temperature to 18°C reduces rainbow trout mortality but has little effect if the fish are already infected, and does not prevent new infections; therefore, the use of elevated water temperature is not practical (Amend, 1970, 1976; Hetrick *et al.*, 1979a). Similarly, infection level in Chinook salmon is influenced by temperature. A temperature of 15°C decreased infection prevalence compared with a temperature of 11°C; however, chronic outbreaks in juveniles occurred at water temperatures in excess of 17°C (Foott *et al.*, 2006).

Fish density is a controllable factor in production facilities and can affect IHNV transmission and prevalence levels. As density increases, fish will be stressed, water quality will likely deteriorate and there is an increased probability of contact between infected animals or infectious agents. Various management strategies, such as holding fish at high density and low flow rates during barging or in spawning channels or tanks, may exacerbate spread of infection unintentionally (Mulcahy *et al.*, 1983a). High densities of spawning fish or crowded eggs and fry are correlated with outbreaks of IHN in fry (Mulcahy and Bauersfeld, 1983; Traxler and Rankin, 1989), possibly due to a rapid horizontal spread of the virus. Under experimental conditions, use of seven densities of rainbow trout and a cohabitation challenge with a single infected fish showed a direct relationship between fish density and transmission of IHNV (Ogut and Reno, 2004b). Chinook salmon smolts had high IHNV concentrations in mucus and showed nipping behaviour when reared at a high density, leading Foott *et al.* (2006) to postulate that this behaviour could act as an avenue of transmission.

Water salinity does not appear to affect the ability of IHNV to infect fish, since experimental bath exposure to IHNV in saltwater has resulted in infection and mortality (Traxler *et al.*, 1993; St-Hilaire *et al.*, 2001, 2002).

The nutritional status of the host, stress and exposure to a polluted environment may influence susceptibility to IHNV. Exposure of rainbow trout to copper increased mortality (Hetrick *et al.*, 1979b). In contrast, exposure of rainbow trout to polychlorinated biphenyls or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin did not affect mortality significantly or mean day to death due to IHNV (Spitsbergen *et al.*, 1988). Exposure of Chinook salmon to two widely used insecticides, chlorpyrifos and esfenvalerate, resulted in altered cytokine expression but did not appear to affect survival negatively after IHNV exposure (Eder *et al.*, 2008).

Immunity

Salmonids mount innate and specific immune responses that result in protection against IHNV. It is now clear that there are three distinct temporal phases: an early antiviral response (EAVR), a specific antiviral response (SAVR) and a long-term antiviral response (LAVR) (Kurath *et al.*, 2006). Since the review of Bootland and Leong (1999), there have been considerable advances in gene sequencing and molecular biology techniques, such as quantitative real-time reverse transcriptase PCR (qRT-PCR) and microarrays (16K VI GRASP chip), which have advanced understanding of fish immune responses to IHNV significantly.

The EAVR or innate non-specific immune response is the first line of defence. This response is induced rapidly within hours to days after exposure to IHNV or vaccination; it has low specificity since it is transiently cross protective against other fish rhabdoviruses and lasts for approximately 3–4 weeks (Kim *et al.*, 2000; LaPatra *et al.*, 2001b; Lorenzen *et al.*, 2002a). This early immunity after viral infection or vaccination is mediated through the type I interferon (IFN) system. Characterization of the EAVR was based initially on the upregulation and production of Mx, a type I interferon (IFN)-inducible antiviral protein, after DNA vaccination (Kim *et al.*, 2000; LaPatra *et al.*, 2001b; Lorenzen *et al.*, 2002a,b). Later studies of the EAVR, using qRT-PCR and microarrays, confirmed an early protective type I IFN response in fish infected with IHNV (Purcell *et al.*, 2004;

Overturf and LaPatra, 2006; Miller *et al.*, 2007; Saint-Jean and Pérez-Prieto, 2007), given a DNA vaccine (Purcell *et al.*, 2004, 2006b) or immunized with a subunit recombinant vaccine (Verjan *et al.*, 2008). The early immune responses after viral infection are complex. Overall, there is upregulation and expression of toll-like receptor (TLR) 8; type I IFN and its inducible genes (e.g. *Mx*, interferon regulatory factors [*IRF-1*, *IRF-2*, *IRF-3*] and *Vig-8*); proinflammatory cytokines (e.g. interleukin IL-1β1, tumour necrosis factors [TNF-α1, TNF-α2]); chemokine IL-8, signalling molecules (e.g. STAT-1) and non-viral specific genes (*Hsp70*, complement factor C3). In a recent study, administration of recombinant Atlantic salmon IFN-α2 by injection, but not by immersion or the oral route, induced dose-dependent short-term protection lasting 1–3 days in rainbow trout and induced the expression of many of the IFN-inducible genes listed above (Ooi *et al.*, 2008). There are also indications of the beginning of a switch from innate to adaptive immunity occurring during the late EAVR phase based on finding upregulation of interferon gamma (IFNg), the IFNg-induced galectin-9 gene, *IRF-2*, which favours an IFNg-induced cellular response, and cytotoxic T-cell marker, CD-8. The strong IFN-like response in infected rainbow trout leads to the early induction of the genes involved in the MHC class I antigen presentation pathway (Hansen and LaPatra, 2002; Purcell *et al.*, 2004; Landis *et al.*, 2008), which presents intracellularly derived antigens to the T-cell TCR$\alpha\beta$/CD-8 complex. The EAVR induced by DNA vaccines is also complex, but somewhat similar to that induced by viral infection. In the first (Purcell *et al.*, 2004) of two studies examining the EAVR of DNA-vaccinated fish, some distinct differences were observed in the spleen of vaccinated fish compared with infected fish, namely that the DNA vaccine induced only Mx-1 and Vig-8, but not TNF-α1, TNF-α2, IL-1β1, IL-8 or Hsp70. In a more comprehensive examination of DNA-vaccinated rainbow trout, including the muscle at the site of injection and three secondary systemic sites, Purcell *et al.* (2006b) found that in muscle tissue, all genes encoding or relating to type I IFN, type II IFN, T- and B-cells and TNF-α were induced. Multiple biological processes including inflammation, infiltration and activation of immune cells were occurring in the muscle. In the kidney and spleen, there was upregulated expression of genes predicted as type I IFN inducible (*IRF-3*, *Mx-1*, *Vig-1* and *Vig-8*), yet IFN-α1 had only a modest induction. TNF-α1 and TNF-α2 gene expression was upregulated significantly at the site of injection, but not in other tissues. As found after virus infection (Miller *et al.*, 2007), there was evidence that a shift from innate to adaptive immunity was under way. Relating to cell-mediated immunity, genes for type II IFN (IFNg), IFNg-inducible respiratory burst (phox p40), TCRβ chain and the cytotoxic T lymphocyte co-receptor (CD-8α) were upregulated at the site of injection, suggesting that CTLs accumulated at the injection site 7 days postvaccination. For detailed information on the EAVR after IHNV infection or DNA vaccination, refer to the two excellent papers by Purcell *et al.* (2004) and Miller *et al.* (2007). Similar to viral infection or DNA vaccination, a recombinant subunit vaccine also induced an early type I IFN response (production of IFN1, Mx-1), production of proinflammatory cytokines (IL-1β1, TNF-α1) and downstream signalling molecules (STAT-1, IRF-1, IRF-2).

The switch from the EAVR to the SAVR is related to the loss of non-specific protection and the temperature-dependent production of neutralizing antibodies (LaPatra *et al.*, 2001b; Kurath *et al.*, 2006). The SAVR begins 3–4 weeks after infection, is potent in inducing strong specific protection and persists for months (Engelking and Leong, 1989a,b; Hattenberger-Baudouy *et al.*, 1989; LaPatra *et al.*, 1993b; St-Hilaire *et al.*, 2002) or vaccination (Corbeil *et al.*, 2000a; LaPatra *et al.*, 2000). Early studies suggested the protective role of neutralizing antibodies against the IHNV G protein (Engelking and Leong, 1989a,b), and this was confirmed by passive immunization (LaPatra *et al.*, 1993b; Corbeil *et al.*, 1999; Traxler *et al.*, 1999). It is during the SAVR phase that the efficacy of most vaccines is tested by challenging

fish at 4–8 weeks postvaccination, often resulting in an RPS of over 90% for DNA vaccines (Anderson *et al.*, 1996a; Traxler *et al.*, 1999; Corbeil *et al.*, 2000a; Kurath *et al.*, 2006). It has been shown repeatedly that serum-neutralizing antibodies are induced by DNA vaccines encoding the IHNV G protein and that these antibodies play a significant role in immunity (Anderson *et al.*, 1996a; Boudinot *et al.*, 1998; Corbeil *et al.*, 1999, 2000a,b; LaPatra *et al.*, 2000; Kurath *et al.*, 2006). However, fish protection can be achieved when only low levels of neutralizing antibodies are detected (Anderson *et al.*, 1996b; Corbeil *et al.*, 199; Traxler *et al.*, 19999). This indicates that antibody levels in vaccinated fish should not be used as the sole criteria for assessing immune status and that antibodies are not the only element of protective immunity. There is increasing evidence of specific cell-mediated immunity after IHNV infection or DNA vaccination, initially suggested during studies of the EAVR (Hansen and LaPatra, 2002; Purcell *et al.*, 2004, 2006b; Miller *et al.*, 2007; Landis *et al.*, 2008). Further research using similar molecular tools and techniques as used to study the EAVR should help elucidate the adaptive immune response during the SAVR.

The third phase, the long-term antiviral response (LAVR), was first described by Kurath *et al.* (2006). This stage begins in rainbow trout at 6 months post-DNA vaccination and lasts for at least 25 months. It is characterized by a reduction in seroprevalence and neutralizing antibody titres, which decline to undetectable levels. Although the level of protection decreases, the RPS remains between 47 and 69%. It was suggested the fish might have had an anamnestic response, with the vaccine acting as an immunological primer. Kurath *et al.* (2006) predicted that the LAVR would have the same high specificity as in the SAVR and that the mechanism of protection would involve additional factors such as cellular immunity.

Many questions still remain regarding immune mechanism(s) involved in the EAVR, SAVR and LAVR. The exact protective mechanisms remain to be identified, but data obtained so far indicate that early non-specific protection is related to interferon, whereas antibodies and cellular components probably both play a role in long-lasting protection.

Epizootiology, Control and Treatment

Epizootiology

The epizootiology of IHNV is still not understood completely but the survival of IHNV in a fish population depends on a close association of the virus with the life cycle of the fish host (Fig. 2.1). IHN outbreaks are observed most often among young salmonids within the first 1–2 months posthatch. Young fish show signs of IHN and mortalities within 5–10 days of exposure (Amend, 1975; Nishimura *et al.*, 1988; Yamamoto *et al.*, 1990; Kim *et al.*, 1999; Troyer *et al.*, 2000) and losses of 80–100% can occur. Once clinical signs become apparent, the disease is usually irreversible and fatal. Vertical transmission may be the cause of infection in some young fish, but IHN can occur during the fry stage or in smolts when a single infected fish infects all other fish horizontally in the rearing unit (Meyers *et al.*, 2003). Horizontal fish-to-fish transmission through the water is the primary means of transmission in all sizes of fish. Although IHN occurs frequently in fry and fingerlings reared in hatcheries, it also occurs in young feral or wild salmon (Traxler, 1986; Traxler and Rankin, 1989). Older salmonids are also susceptible to IHN mortality. Yearling and commercial-sized rainbow trout (Busch, 1983; Nishizawa *et al.*, 2006), yearling Chinook salmon, sockeye salmon smolts (Yasutake, 1978; Burke and Grischkowsky, 1984) and 2- to 3-year-old wild kokanee salmon (Traxler, 1986; Anderson *et al.*, 2000) have succumbed to IHNV in fresh water. In many cases, infected fish within the population or other species of infected fish are thought to be the viral source, and horizontal transmission through the water probably occurs (Burke and Grischkowsky, 1984). IHNV can be prevalent among

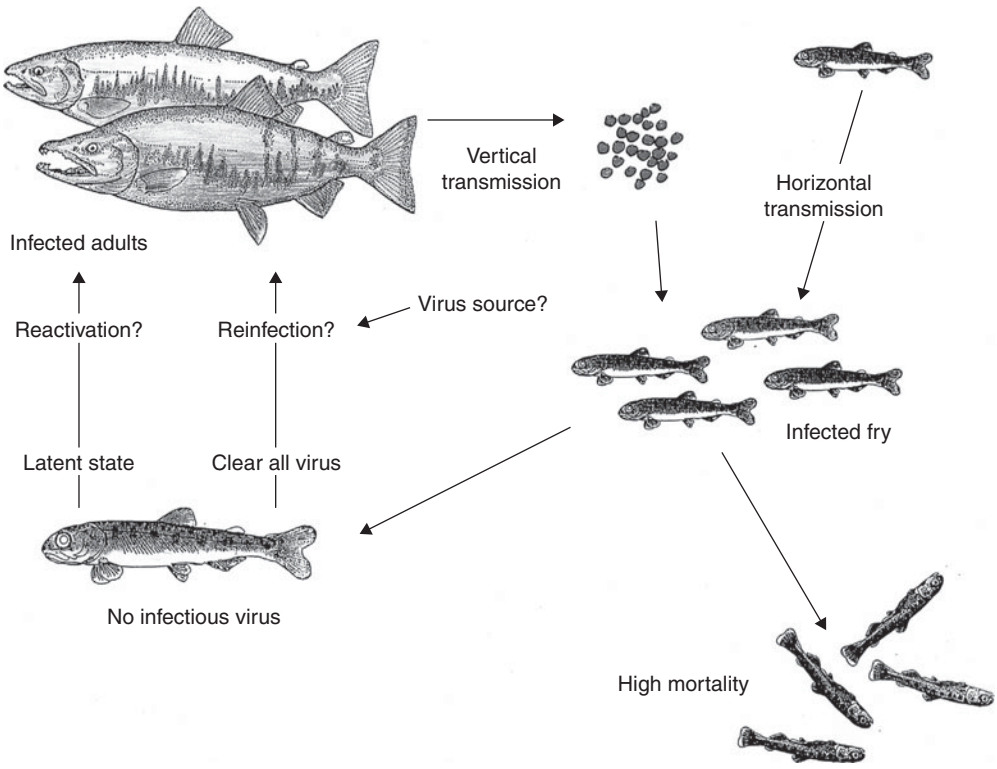


Fig. 2.1. Life cycle of IHNV in salmonid fish.

juveniles in hatcheries and survivors or infected fish may be released and begin their seaward migration.

In California, IHNV was isolated from infected Chinook salmon smolts trapped 295 km downstream at 15 days post-release. Yet, based on laboratory trials, the chance of horizontal transmission of the Sacramento River strain of IHNV from hatchery fish to wild fish was considered of low ecological risk (Foott *et al.*, 2006). Horizontal transmission in salt water was confirmed (Traxler *et al.*, 1993; St-Hilaire *et al.*, 2001) and Atlantic salmon smolts suffered high IHN-induced mortality in marine net pens (St-Hilaire *et al.*, 2002; Saksida, 2006).

What has intrigued biologists most about the life cycle of IHNV is that infectious virus cannot be isolated from surviving salmonids within 2 months after an IHN epizootic or during the outward migration to the ocean, yet virus is again re-isolated when adults return to spawn and reach sexual maturity. There are two hypotheses to explain this phenomenon. The first hypothesis proposes that some fraction of salmonids surviving IHNV infection may become lifelong carriers, with persistent or latent infections that are reactivated as fish become immunocompromised by the stress of the spawning migration and reaching sexual maturity. The initial evidence that IHNV entered a latent state with reactivation was that IHNV could not be re-isolated from rainbow trout within 21 days or over the following 2 years when fish were maintained in virus-free water, yet sexually mature fish had IHNV in the reproductive products (Amend, 1975). However, other studies were unable to detect a latent infection. Fish captured over 3 years from a population which normally had a 98–100%

incidence of IHNV did not become infected when held to sexual maturity in pathogen-free water (Amos *et al.*, 1989). Similarly, it was reported initially that sockeye salmon held in freshwater net pens before the fish reached their spawning grounds did not become infected with IHNV, even though 89% of fish removed from the spawning ground were infected (Kent *et al.*, 1988; Elston *et al.*, 1989). When this study was continued, at least 25% of the fish held in net pens became infected, and yet groups of spawning fish held in two locations upstream of the net pens were uninfected or had an infection rate of 17% (Elston *et al.*, 1989). Experimental support for a carrier state with a latent infection was found when viral proteins, IHNV nucleic acid and potentially biologically active truncated viral particles resembling rhabdovirus defective interfering particles (DIPs) were found in kidney, spleen and liver tissues of rainbow trout survivors of an IHNV epizootic 1–2 years after infectious virus was no longer detectable (Chiou *et al.*, 1995; Drolet *et al.*, 1995; Kim *et al.*, 1999). In a shorter-term study, it was postulated that the strong humoral immune response of rainbow trout could have cleared the virus, since IHNV could not be detected in kidney or brain homogenates of surviving fish sampled for 4.5–12.5 weeks post-infection. This suggests that a latent state in rainbow trout does not occur commonly (LaPatra *et al.*, 2001a). A carrier state may also exist in Chinook salmon based on asymptomatic smolts with a low or undetectable level of infectious virus transmitting IHNV successfully to cohabiting Atlantic salmon (St-Hilaire *et al.*, 2001). It is now clear that at least some IHNV survivors harbour a latent infection but, to date, it has not been possible to show a reactivation of the latent state. The conditions that trigger reactivation still have to be identified.

The second hypothesis proposes that surviving juvenile fish clear the virus completely, but adults become re-infected by a new exposure to IHNV prior to or during the spawning migration. This hypothesis was based initially on the observation that IHNV could not be isolated from sexually mature sockeye salmon during their migration from the ocean to the spawning area, but virus suddenly appeared in a low percentage of pre-spawning females in the spawning grounds. As the spawning migration progressed and fish density increased, viral prevalence and titres increased (Mulcahy *et al.*, 1982, 1984a; Mulcahy and Pascho, 1986). IHNV probably does not survive in water over the winter (Mulcahy *et al.*, 1983b), but titres of over 10^3 pfu/ml have been detected in salmon spawning-streams (Mulcahy *et al.*, 1983a), and these titres are sufficient to infect salmonids. Adult salmonids are susceptible to infection with IHNV from exogenous sources in both fresh water (Yamamoto *et al.*, 1989; LaPatra *et al.*, 1993b; Arkush *et al.*, 2004) and salt water (Traxler *et al.*, 1993). Arkush *et al.* (2004) supported that adult salmonids became infected via virus present in the spawning environment after observing that an immersion challenge led to a rapid progression of infection in sexually mature Chinook salmon with the release of high concentrations of virus in ovarian fluids. Transmission of the virus among overlapping runs of adult salmon or infections among landlocked or resident salmonids could be a source of virus maintenance in fresh water and could result in infection of wild and hatchery-reared adult salmonids (Arkush *et al.*, 2004; Kelley *et al.*, 2007).

IHNV transmission does occur in the marine environment (Traxler *et al.*, 1993), even though IHNV survives poorly in salt water, with only 0.1% surviving after 2 weeks at 15°C (Pietsch *et al.*, 1977; Toranzo and Hetrick, 1982). A significant finding was that IHNV could be isolated from adult sockeye salmon during the ocean phase of their life cycle and that the fish could have been exposed 5–6 months prior to sexual maturation (Traxler *et al.*, 1997). It has been suggested that sockeye salmon populations may harbour a high percentage of carriers that presumably shed virus while migrating to spawning areas (Amend, 1975; Traxler *et al.*, 1997). The annual return of wild sockeye salmon coincided with some epidemics in Atlantic salmon in marine net pens and it was suggested that wild salmon

were the initial source of infection and then the virus spread horizontally within and between pens (St-Hilaire *et al.*, 2002; Saksida, 2006).

Rudakova *et al.* (2007) found a lack of evolutionary divergence between IHNV isolates from sockeye salmon in Russia and the USA and suggested that fish were infected by direct transmission with a common source of IHNV in the Pacific Ocean, or existence of a non-sockeye marine reservoir. A marine reservoir in the Pacific Ocean has not been confirmed. Potential vectors are ectoparasites, since IHNV has been isolated from copepods (*Salmincola* sp.) removed from sockeye salmon (Mulcahy *et al.*, 1990). Marine non-salmonid fish may be a reservoir of infection as IHNV has also been isolated from several non-salmonid cold-water marine finfish species (Kent *et al.*, 1998).

Control and treatment

Current IHNV control strategies have not changed significantly since the previous review (Bootland and Leong, 1999) and still rely principally on good hatchery practices. Control measures for IHN currently rely on avoidance of exposure to the virus through the implementation of strict control policies and sound hygiene practices (OIE, 2006). A 2007 fact sheet (Spinkler, 2007) indicates that, in areas where the disease is not endemic, outbreaks are controlled by culling, disinfection, quarantine and other measures. Where IHNV is endemic, good biosecurity and sanitation decrease the risk of introducing the virus to a farm. Additional precautions generally accepted in British Columbia salmon farming are maintaining a single year class and single species sites, reduced fish movement between pens, culling/accelerated harvest of smaller fish if infection occurs and fallowing between re-stocking (Saksida, 2006). Introduction of IHNV into new geographical areas has never been associated with the movement of killed fresh fish or frozen product and the risk is considered negligible for processed rainbow trout (LaPatra *et al.*, 2001a).

There are no approved chemotherapeutic agents to treat or prevent IHN and only a few new compounds have been evaluated since the review of Bootland and Leong (1999). In cell cultures, IHNV replication is inhibited by methisoprinol (Siwicki *et al.*, 2002). Chloroquine is effective *in vivo* (Hasobe and Saneyoshi, 1985), as this compound reduces IHNV binding and cell entry (De las Heras *et al.*, 2008). Similarly, pretreatment of cells with the antiviral agents, amantadine or tributylamine, also reduces IHNV binding and cell entry (De las Heras *et al.*, 2008). Antisense phosphorodiamidate morpholino oligomers (PMO), a new class of antisense agents, were first tested against IHNV by Alonso *et al.* (2005). PMO inhibits IHNV replication in cell culture by acting on the IHNV RNA polymerase or nucleoprotein. PMO is taken up by tissues when fish are exposed to it via immersion. The efficacy of the above compounds against IHNV has not been tested *in vivo*.

Selective breeding of salmonids for increased resistance against IHN is a potential control strategy. Interspecific hybrids with enhanced resistance have been made between susceptible and resistant species; for example, rainbow trout crossed with coho salmon, cutthroat trout or brook trout (Parsons *et al.*, 1986; Chen *et al.*, 1990; Dorson *et al.*, 1991; LaPatra *et al.*, 1993c). Hybrids generally have poorer performance in culture compared with parental species, but are useful for examining resistance mechanisms (LaPatra *et al.*, 1996). Attempts are being made to understand the host genetic elements responsible for IHNV resistance and although it is clear that numerous genes are involved, specific genes have not yet been identified. The majority of genetic studies on resistance to IHNV have been on rainbow trout backcrossed within families (Khoo *et al.*, 2004) or with less susceptible cutthroat trout (Palti *et al.*, 1999, 2001; Barroso *et al.*, 2008) or steelhead trout (Rodriguez *et al.*, 2004). Genetic linkage maps were produced based on molecular markers (microsatellites and amplified fragment length polymorphisms (AFLP)) and

quantitative trait loci (QTL) were identified. DNA markers coupled with physical mapping of the trout genome should enable future positional cloning of the loci and, ultimately, identification of the genes responsible for IHNV resistance (Barroso *et al.*, 2008).

Vaccines

A major breakthrough was achieved in July 2005 with the licensing of the first DNA vaccine for fish. APEX-IHN (Novartis Animal Health Canada Inc.) was approved for market by the Canadian Food Inspection Agency (CFIA) for the prevention of IHN in farm-raised Atlantic salmon. This momentous achievement came after years of research and development by numerous scientists from academia, government and industry. Pioneering research showed that an intramuscular injection of plasmid DNA encoding the IHNV *G* gene induced protection in rainbow trout (Anderson *et al.*, 1996a,b) and also in Atlantic salmon smolts (Traxler *et al.*, 1999). Since then, numerous experimental studies by researchers in the USA have confirmed the efficacy of a similar DNA vaccine (pIHNw-G encoding the *G* gene from the IHNV WRAC strain) and have examined several practical aspects such as vaccine dose, duration of protection, cross-species protection, routes of administration and vaccine safety. It was confirmed that the DNA vaccine must encode the IHNV *G* gene to induce protection and neutralizing antibodies in rainbow trout fry and an antibody response in sockeye salmon (Corbeil *et al.*, 1999). The vaccine was effective at extremely low doses (0.1–1 μg) in 1–2 g rainbow trout fry (Corbeil *et al.*, 2000a; LaPatra *et al.*, 2001b) and a standard effective dose of 0.1 μg induced protection against a broad range of rainbow trout IHNV isolates from different geographical locations, suggesting that the DNA vaccine would be useful worldwide (Corbeil *et al.*, 2000a). Larger fish require a higher dose of the vaccine to induce protection and, as a rough guideline, an intramuscular dose of 10 ng/g body weight is required (Lorenzen *et al.*, 2002b). The DNA vaccine induces rapid innate immunity followed by long-term specific protection; RPS >90% for at least 3 months then reduced but still significant levels of protection (RPS typically >60%) for as long as 2 years after vaccination (Kurath *et al.*, 2006). The DNA vaccine protects numerous salmonid species, including Atlantic salmon (Traxler *et al.*, 1999), rainbow trout (e.g. Corbeil *et al.*, 1999, 2000a; Kurath *et al.*, 2006), Chinook salmon, sockeye salmon and kokanee salmon (Garver *et al.*, 2005a). All of the above studies used intramuscular (i.m.) injection of the DNA vaccine, but this is labour-intensive and is not very practical for the mass immunization of small fish. There was good protective immunity in rainbow trout fry when the vaccine was given by intramuscular injection or cutaneous particle bombardment using a gene gun and partial protection by intraperitoneal injection, but other routes including scarification of the skin, intrabuccal administration or immersion in DNA-coated polystyrene beads did not induce protection (Corbeil *et al.*, 2000b). An interesting alternative route for mass immunization was investigated by Kurath and Pascho (2005), where the DNA vaccine was added to water during egg hardening. Although this route did not induce tolerance, it also did not elicit any protective immunity in progeny fry.

The IHNV DNA vaccine has been shown to be safe and well tolerated by fish since there were no vaccine-specific pathologic changes in any tissue at 90 days or as long as 2 years after vaccination (Garver *et al.*, 2005b; Kurath *et al.*, 2006). The plasmid backbone in the above studies contained the cytomegalovirus (CMV) immediate early promoter, which was derived from a human pathogen. Since regulatory authorities may consider such a vaccine 'unsafe', Alonso *et al.* (2003b) evaluated the use of plasmid DNA containing the IHNV *G* gene linked to rainbow trout interferon regulatory factor 1A (pIRF1A-G), MxSpeI (pMxSpeI-g) or Mx (pMX-G) promoters as a vaccine for rainbow trout fry. Fish challenge at 30 days resulted in <50% mortality in the negative control groups, but the pIRF1A-G induced significant protection (RPS 55%), which was equivalent to the protection induced by the standard pCMV-G

vaccine. The mechanism of protection was not evaluated, but the results provided evidence that promoters other than CMV could be used in fish DNA vaccines.

Given the great economic need for an IHNV vaccine in British Columbia and the very promising safety and efficacy of experimental IHNV DNA vaccines, a team at Novartis Animal Health Canada Inc. (formerly Aqua Health Ltd) addressed several critical issues such as regulatory and environmental concerns, risk–benefit, feasibility of producing the vaccine at a scale and cost appropriate for the fish industry and intellectual property during the development and licensing of APEX-IHN (reviewed by Salonius *et al.*, 2007). The CFIA (2005) document provides details on the recombinant plasmid containing the IHNV *G* gene from a BC sockeye salmon isolate (pUK-ihnG), human and animal safety and environmental assessment for licensing of APEX-IHN in Canada, and is available online. As required for any licensed vaccine, the safety, purity, efficacy and potency of the product were confirmed. The label instructions indicate that the vaccine is administered to Atlantic salmon by intramuscular injection at least 400 degree-days prior to seawater transfer. Safety testing in the field used a total of 6 million doses to vaccinate farmed Atlantic salmon at seven sites/hatcheries on the British Columbia coast. No adverse events in the farmed salmon related to the use of APEX-IHN were reported at any of the sites. Typically, salmon are vaccinated by intraperitoneal injection with polyvalent oil-adjuvanted vaccines. It was confirmed experimentally that administration of APEX-IHN by intramuscular injection with concurrent intraperitoneal vaccination with a multivalent bacterin vaccine did not affect the efficacy of the DNA vaccine (Salonius *et al.*, 2005). Large-scale field efficacy testing of APEX-IHN was initiated in British Columbia during the licensure process, with more than 3 million salmon vaccinated; however, a natural IHNV challenge did not occur to demonstrate strong field efficacy. To obtain efficacy data, laboratory trials using elevated levels of IHNV were used in cohabitation challenges, resulting in 79–100% RPS (MacKinnon *et al.*, 2008).

Although there is now a commercial DNA vaccine for smolt-sized fish, a cost-effective and efficacious method for the mass immunization of small fish is needed urgently. As DNA vaccination of fish is a relatively new technology, there are a number of consequences and uncertainties, many of which have been identified in the survey by Norwegian scientists (Gillund *et al.*, 2008). Even though APEX-IHN has been licensed in Canada and many of the safety and environmental issues are addressed (CFIA, 2005; Salonius *et al.*, 2007), there are regulatory and public perception issues to overcome before this vaccine or other DNA vaccines are licensed in other countries. There is a lack of clarity in distinguishing DNA-vaccinated fish from genetically modified organisms (GMO); hence, vaccinated fish may be labelled as GMO, which may affect the market price via the consumer's willingness to buy DNA-vaccinated fish (Gillund *et al.*, 2008). Further research addressing theoretical and long-term environmental and consumer safety issues, in conjunction with public education, is critical to acceptance of DNA vaccines for fish.

Extensive efforts have been made to develop conventional killed vaccines, subunit vaccines and attenuated vaccines. Experimental IHNV vaccines reviewed in Winton (1997) and Bootland and Leong (1999) showed varying levels of efficacy and have not resulted in a commercially viable product. However, research efforts are still continuing. A re-examination of killed IHNV vaccines was completed by Anderson *et al.* (2008) and drastic effects of virus-inactivating agents on induction of protection in rainbow trout were observed. The most efficacious vaccine was β-propiolactone (BPL) inactivated whole virus, which induced both short-term (7 days) and long-term protection (56 days). Formaldehyde, a common inactivating agent, yielded inconsistent efficacy, and virus inactivated by heat or binary ethylenimine (BEI) were not efficacious. Hence, there is still the potential to develop an effective killed vaccine.

There are three recent studies examining recombinant subunit vaccines. A

baculovirus-derived recombinant IHNV G protein vaccine produced in insect cells did not result in significant protection in rainbow trout, which might have been due to limited neutralizing antibody production (Cain *et al.*, 1999). Similarly, a recombinant subunit vaccine based on the production of a soluble 184 amino acid sequence of IHNV G fused in different arrangements to the bacterial *Caulobacter crescentus* S-layer protein induced only limited protection (RPS < 35%), as did BEI inactivated whole virus emulsified in mineral oil adjuvant (RPS < 38%) (Simon *et al.*, 2001). A subunit vaccine containing purified soluble non-glycosylated IHNV G protein expressed in *E. coli* BL21 cells using the pET-32a(+) vector stimulated early innate protection in rainbow trout (Verjan *et al.*, 2008). This vaccine is promising, but administration by a route other than intraperitoneal injection and evaluation of longer-term protection are required.

Live attenuated IHNV vaccines have shown promise recently. Through the use of reverse genetics, recombinant IHNV containing a deletion of the *NV* gene resulted in irreversible attenuation of virus virulence and induction of very high levels of protection in rainbow trout when the virus was administered by injection or immersion (Thoulouze *et al.*, 2004). In further studies, recombinant IHNV generated by replacement of the *NV* gene with green fluorescent protein (rIHNV-GFP) or substituting the IHNV *G* gene with the *G* gene of VHSV (rIHNV-Gvhsv GFP) induced heterologous protection in rainbow trout (Romero *et al.*, 2008). Fish showed an RPS of 70% and 62% against IHNV and 49% and 61% against VHSV using the rIHNV-GFP and rIHNV-Gvhsv GFP vaccines, respectively. Neither vaccine induced more than a minimal antibody response over 10 weeks, or early (1–3 days) expression of the type I IFN or interferon-induced (*Mx*, *ISG-15*) genes. RT-PCR did not detect replication of the recombinant viruses by day 3. The mechanism of protection remains elusive and requires further research. Although these live vaccines have given promising experimental results, the feasibility and cost for scaling up vaccine production to commercial levels, vaccine safety and other regulatory concerns will have to be addressed.

Conclusions and Recommendations for Future Studies

In 1999, we recommended research that would determine how IHNV carrier states were maintained in fish and how the virulence of the virus was altered for different host species. Only a full understanding of the entire life cycle of the virus and its interactions with the host cell and the host organism will lead to effective measures of controlling the spread of IHNV in cultured and wild fish. In 2003, a workshop sponsored by the Science Council of British Columbia, British Columbia Ministry of Agriculture, Food and Fisheries, developed a white paper outlining the IHN Research and Management Needs. The workshop participants identified four major research priorities: diagnostic test validation; transmission and source of IHNV in outbreaks; vaccine design; and epidemiological studies. The immediate research questions are: (i) can early detection or prediction of IHN be achieved in a manner that allows for action to prevent or reduce the impact of disease?; (ii) can a weak point in the transmission cycle between farmed fish and/or their environment be found?; and (iii) can a safe and efficacious vaccine be developed within the current regulatory framework? The ultimate research aim was the reduction of the impacts of IHN disease on salmon farms. Significant progress has been made in some of these areas. Rapid, sensitive molecular diagnostic tests have been developed and strides have been made in understanding the early immune response against IHNV. The first licensed DNA vaccine protects against IHNV, but an alternative to injection administration is needed. In 2008, the research priorities remain largely the same as those outlined in the white paper. These are difficult questions and require resources for testing vaccines under controlled conditions and field studies that require considerable personnel investments.

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3 Viral Haemorrhagic Septicaemia

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Introduction

In 2009, viral haemorrhagic septicaemia (VHS) was recognized as a disease of both farmed salmonid and non-salmonid fish, plus a great range of wild fish, with a global distribution throughout the northern hemisphere. VHS is still the most serious viral disease of farmed rainbow trout (*Oncorhynchus mykiss*) in Europe and is responsible for significant losses in the Scandinavian countries, for example, Norway and Sweden, as well as the Continental countries, for example, Germany and Italy. To date, the global economic costs of losses due to VHS have not been quantified accurately, for this is a gap in research (see the section below on economic importance). VHS still poses a threat to farmed trout because of the carrier state of VHS virus (VHSV) in wild marine stocks in water systems close to fish farms, as first highlighted by Meier *et al.* (1994).

The pathogen is a rhabdovirus belonging to the family *Rhabdoviridae*, the same virus family as rabies virus of dogs and foxes. Some pronounced features of the virus are the ability to cause marked kidney necrosis, especially of the head region, which causes loss of haematopoietic cells important for constitutive and adaptive immunity. In the later stages of the disease, there can be tropism of the brain tissue, a feature shared with other rhabdoviruses. The disease was first described in rainbow trout by Schaperclaus (1938) and a viral aetiology was postulated. It was not until 1963 that Jensen isolated a European strain of the virus and over 20 years later before a Pacific marine strain of VHSV was reported from the USA (Brunson *et al.*, 1989). Characterization of the virus and its effects proceeded rapidly, particularly with pathogenicity and vaccine studies in France and Denmark (de Kinkelin, 1988; Wolf, 1988).

Viral haemorrhagic septicaemia is still the most important disease of cultured rainbow trout in the European Union (EU) member states and no universal vaccine has been developed to date, despite considerable effort. The most effective control therefore remains avoidance.

The Disease Agent

Current classification

The viral haemorrhagic septicaemia virus is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_rhabd.htm). The type species of this genus is the related fish virus infectious haematopoietic necrosis

© CAB International 2011. *Fish Diseases and Disorders* Vol. 3: *Viral, Bacterial and Fungal Infections*, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

virus (IHNV). Members of this genus share a common and distinctive feature (the presence of an additional non-virion protein encoding gene) within the *Rhabdoviridae*.

Biology

Morphology and structure of the virion

Rhabdoviruses are bullet-shaped viruses, typically measuring approximately 200×75 nm (Coll, 1995a). VHSV displays helical symmetry, with particle measurements varying from 165×75 nm (Olberding and Frost, 1975) to 240×75 nm (de Kinkelin and Scherrer, 1970). The variation likely reflects different preparative conditions for electron microscopy rather than real strain differences in size. The particle comprises a helical core of single-stranded RNA, stabilized by matrix protein, to which are bound major structural proteins, and the nucleocapsid is surrounded by an outer lipoprotein layer. In ultrathin sections, an electron-lucent central axis 20–35 nm wide is observed and externally there is an additional electron-dense layer which represents the envelope (Baroni *et al.*, 1982). Parallel striations on the exterior of the envelope are seen by negative staining and evidence the helical symmetry of the particle.

The structure of the VHSV virion is assumed to be similar to other rhabdovirus models such as rabies virus, the type species of the *Lyssaviridae*. The VHSV virion is comprised of five proteins: a phosphorylated nucleoprotein (N; 38 kDa), a polymerase associated protein (P; 24 kDa), a matrix protein (M), a glycoprotein (G; 65 kDa) and an RNA polymerase (L; 200 kDa) (Estepa *et al.*, 1994; Basurco and Benmansour, 1995). The structural proteins are arranged to form a helically wound ribonucleocapsid core which is surrounded by a lipid bilayer envelope through which glycoprotein spikes project. The glycoprotein of VHSV forms homotrimer spikes: this is the major neutralizing surface antigen which is responsible for attachment and entry into host cells and has been shown to be an important molecule in eliciting host immune responses (Lorenzen, N. *et al.*, 1999). The ribonucleocapsid core provides the transcriptase activity of the virus and is composed of ribonucleoprotein (RNP) (single-stranded RNA genome N and M protein in association with L protein). The matrix protein (M) lines the viral envelope and interacts with the cytoplasmic domain of the surface glycoprotein. Indeed, the M protein has been demonstrated to attach both to the G on the internal side of the membrane and to the ribonucleocapsid (demonstrated by cross-linking) on the opposite side (Zakowsky and Wagner, 1980; Kaptur *et al.*, 1991; Chong and Rose, 1993). The M protein, including that of the fish rhabdoviruses (Benmansour *et al.*, 1994), is basic (Li *et al.*, 1993), most probably to attach to the negatively charged residues on the inner surface of the membrane (Zakowsky and Wagner, 1980).

Viral life cycle

The typical viral replication cycle consists of five main stages.

ADSORPTION. The glycoprotein is responsible for the attachment of virus to the cellular membrane receptors, and a specific receptor has been identified in Salmonidae-derived cell monolayers which can be blocked by a receptor-directed MAb (M45) in order to prevent infection by fish rhabdoviruses (Béarzotti *et al.*, 1995b).

PENETRATION AND UNCOATING. Penetration of host cells and the uncoating of enveloped virions closely follow adsorption, and the two events are not readily dissociable. Granzow *et al.* (1997) have demonstrated that the internalization of fish rhabdoviruses VHSV, IHNV and spring viraemia of carp virus (SVCV) adsorbed to the plasma membrane occurs by receptor-mediated endocytosis. Following endocytosis, the coated vesicles fuse with primary lysosomes, resulting in the release of nucleocapsid, which can accumulate to form cytoplasmic inclusion

bodies, indicating the first signs of VHSV infection (Granzow *et al.*, 1997).

TRANSCRIPTION AND TRANSLATION. Transcription is the first metabolic event to occur following the release of uncoated nucleocapsids into the cytoplasm. The genomic negative polarity RNA is transcribed sequentially and attenuation at each gene junction results in a decrease in the amount of messenger RNA (mRNA) synthesized, depending on the distance from the 3′-terminal start site of transcription (Rose and Schubert, 1987). Translation of the mRNAs of vesicular stomatitis virus (VSV) is coupled to transcription, with all proteins being synthesized throughout the cycle of infection (Wagner, 1987).

REPLICATION. Replication results in the production of full-length complement of the minus-strand genomic RNA. The polymerase must act in a processive mode for synthesis of full-length RNA, in addition to a non-processive mode for synthesis of individual mRNAs destined for translation into viral proteins in which internal genome signals define start and stop sites (Conzelmann, 1996).

ASSEMBLY AND BUDDING. In the case of VSV, replication of viral progeny and translation proceed simultaneously and the newly synthesized N, L, P and M proteins bind to the viral genomic RNA to form nucleoprotein cores (Rubio *et al.*, 1980). Throughout these events, VSV G protein is synthesized independently on membrane-associated polyribosomes and processed and glycosylated in endoplasmic reticulum-Golgi structures. Coated vesicles then migrate to the plasma membrane for fusion and insertion of the G protein (Rothman *et al.*, 1980). The nucleocapsid M protein appears to bind to the cytoplasmic surface of the plasma membrane at a region rich in inserted G protein and phospholipids. The highly coiled nucleocapsid is next enveloped in the G protein-covered cytoplasmic membrane, leading to the budding and release of infectious virions (Wagner, 1987).

Phenotypic and antigenic characteristics

Forms of the virus

In common with many RNA viruses with a negative-strand polarity genome, defective interfering (DI) particles are readily produced which are shorter and have a smaller RNA molecule than normal infectious particles. Such particles interfere with the standard complete infectious virus at the cellular level by competing for cell receptor binding sites, and thereby blocking efficient replication of the standard full-length virus (Wunner, 1985).

Serotypes and strains

Three serotypes of VHSV have been described: type 1, represented by strain Fl (Denmark); type 2, represented by the Heddedam isolate from Denmark; and type 3, represented by the French strain 23/75 isolated from brown trout (*Salmo trutta*) by de Kinkelin and Le Berre (1977). These three serotypes were defined by a serum neutralization test. However, as Olesen *et al.* (1993) reported, there was considerable overlap of strains within and between these serotypes. These authors attempted to define more realistic serogroups, using cross-reacting polyclonal antiserum and four neutralizing monoclonal antibodies (MAbs). It was suggested that three serogroups were recognizable: serogroup I, neutralized by all four MAbs and the anti-Fl antibody; serogroup II, neutralized by only one MAb and the anti-Fl antibody; and serogroup III, not neutralized by any MAb but with low or no neutralization by the Fl antibody.

Although three serogroups were identified, Olesen *et al.* (1993) pointed out that 120 out of 127 isolates were neutralized by an anti-Fl antiserum and these therefore showed common antigenic determinants. Serogroups were therefore, in reality, subtypes within a single serotype and the definition of the subtypes would be dependent on the MAbs used. What emerged from the study of newly classified VHS viruses by Olesen *et al.* (1993) was that there had been

an antigenic shift from the period 1965–1981 to 1992 in Denmark, by which the group I isolates, including Fl type isolate, had been largely displaced by new group II isolates in a single year from 1991 to 1992. What is known about marine VHSV strains in European waters from cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) in the North Sea and turbot (*Psetta maxima*) from Gigha Island, Scotland is that all the three neutralization serogroups are represented.

Currently, neutralization patterns for new VHSV isolates using the above MAbs are rarely reported, an exception being the characterization of new Finnish isolates reported as type I pattern (Raja-Halli *et al.*, 2006). The characterization of serotype- and subtype-specific antigens has been investigated widely. However, programmes for genetic analysis can compute the degree of nucleotide similarity between viral genes, leading to classification of isolates within distinct genetic groups or genotypes. This type of analysis is more informative and robust, and thus more suited to classifying VHSV types on an evolutionary basis than MAb neutralization patterns.

Genotypes

Four main genotypes of VHSV have been defined based on the phylogenetic analysis of nucleotide sequence data sets. These genotypes are consistent, robust and independent of the gene sequences used for classification (Snow *et al.*, 2004; Einer-Jensen *et al.*, 2005). These genotypes show a geographic basis for their distribution rather than host specificity.

Geographic Distribution and Host Range

Geographical distribution and origins of VHSV

Genotype I (GI) includes VHSV isolates in circulation in rainbow trout farms in Continental Europe which are generally associated with clinical disease and high economic loss (indicated as Genotype Ia; GIa). Genotype Ib comprises marine isolates from the Baltic Sea, Skaggerak and Kattegat, the North Sea and the English Channel. Genotype Ic consists of a small group of historical Danish isolates from freshwater rainbow trout. Genotype Id consists of a small group of isolates from rainbow trout in the brackish waters of the Gulf of Finland (Raja-Halli *et al.*, 2006). Genotype Ie consists of isolates from free-living turbot off the Turkish coast of the Black Sea (Nishizawa *et al.*, 2006). The distribution of Genotype Ia has no doubt been influenced heavily by anthropogenic factors associated with fish farming activity. Indeed, the virus responsible for the first recorded outbreak of VHSV in the UK rainbow trout sector in 2006 was characterized as GIa, suggesting likely import of this virus from a VHSV infected area within Europe (Stone *et al.*, 2008). Interestingly, GI also includes isolates recovered from a wide range of wild caught asymptomatic fish species caught largely in the Baltic Sea. This has led to speculation that VHSV was introduced into rainbow trout farms originally from the Baltic Sea, possibly through the once common practice of using unpasteurized wild fish products as feed in the aquaculture industry (Dixon, 1999). In experimental trials, Genotype Ib isolates from wild marine fish species generally do not cause clinical disease in rainbow trout; this suggests some kind of virulence shift or adaptive evolution in association with the introduction. Such a shift in virulence seems to have occurred on multiple independent occasions with outbreaks of VHS in marine rainbow trout farms that have been classified within further subgroups of Genotype I (Einer-Jensen *et al.*, 2004). Interestingly, Genotype Ib isolates have been identified outside the Baltic Sea area, in herring (*Clupea harengus*) caught in the English Channel (Dixon *et al.*, 1997) and east of the Shetland Isles (King *et al.*, 2001). This could be a reflection of the migratory habits and population structuring of this species.

Genotype II isolates have been recovered from wild fish only in a limited area of the Baltic Sea, the Gotland Basin. Their genetic isolation suggests that the main genotypes separated many years ago, which lends support to the theory of a marine origin of VHSV.

Genotype III includes isolates from wild fish caught in the North Sea and the eastern and western Atlantic. Viruses responsible for disease outbreaks in cultured turbot have also been assigned to this genotype. Turbot have been shown to be experimentally susceptible to a number of naturally present GIII isolates (Snow and Smail, 1999; Snow *et al.*, 2005).

Genotype IV isolates were first discovered in the Pacific North-west but subsequently have been identified in eastern USA, the Great Lakes region and Japan. The fact that VHSV has not been identified in rainbow trout in the USA, coupled with the genetic isolation of GIV, further supports a theory for a marine origin of VHSV. The virus, which apparently has spread recently to the Great Lakes region, has been classified in a separate subgroup, GIVb (El sayed *et al.*, 2006; Gagne *et al.*, 2007; Lumsden *et al.*, 2007). The possible introduction of this virus into a potentially new environment has caused epizootics in a range of likely previously naive wild fish species.

With respect to genotype/virus isolate and sequence information, a user-friendly virus isolate and sequence database has been set up recently as a public website, http://www.FishPathogens.eu/vhsv (Jonstrup *et. al.*, 2009), and is maintained by the Community Reference Laboratory for Fish Diseases.

Transmission including developmental stages

VHSV is a highly transmissible virus and, in experimental infection trials, the virus can be transmitted by intraperitoneal injections, bathing and cohabitation. For example, López-Vásquez *et al.* (2007) tested the virulence of a wild Greenland halibut (*Reinhardtius hippoglossoides*) isolate of VHSV to turbot. In the main, VHS virus is more pathogenic to sac fry, fry and fingerlings than to older grower fish. Viral shedding via the urine has been demonstrated in clinically diseased rainbow trout (Neukirch, 1985) and horizontal transmission can therefore take place via this route.

Host range

Understanding the host range of VHSV has changed markedly in the past 10 years as monitoring studies have expanded across the northern hemisphere. In the first edition of this chapter, 17 host species of teleost fish were listed. Skall *et al.* (2005) listed 53 species of marine and freshwater fish from which VHSV had been isolated and 11 fish species as experimentally susceptible, giving a total of 64. The International Database on Aquatic Animal Disease (http://www.collabcen.net/idaad) lists 63 host species as either natural or experimental hosts of VHSV. An updated listing of VHSV susceptible hosts is given in Table 3.1.

Rainbow trout are especially susceptible to VHSV and the classical signs are seen in first-feeding fry. This virus was imported to Europe, being transported from the USA in the last century. Speculating whether the disease would have become established is interesting if rainbow trout had not been imported from the USA first into France in 1879 and then later into Denmark. Rainbow trout in seawater cages are also susceptible; Castric and de Kinkelin (1980) reported an outbreak where 85% mortality was observed 80 days after seawater transfer.

Other species of trout are susceptible to VHSV under natural conditions, including brook trout (*Salvelinus fontinalis*) (Rasmussen, 1965) and lake trout (*S. namaycush*) (Ghittino, 1973). Within the genus *Salmo*, Atlantic salmon (*S. salar*) (Rasmussen, 1965), brown trout and golden trout (*S. aguabonita*) are susceptible to VHSV. Wild freshwater inhabitants of streams, rivers and lakes in Germany and Switzerland are susceptible, notably pike (*Esox lucius*), grayling (*Thymallus thymallus*) and

Table 3.1. Susceptible hosts of VHSV.

Host Species	Specific name	Reference
Atlantic cod	*Gadus morhua* L.	Jensen *et al*. (1979)
Atlantic herring	*Clupea harengus*	Dixon *et al*. (1997)
Atlantic halibut	*Hippoglossus hippoglossus*	Bowden (2003)
Atlantic salmon	*Salmo salar*	OIE (2005)
Barbel (Spanish subsp.)	*Barbus graellsi*	Basurco and Coll (1989)
Black crappie	*Pomoxis nigromaculatus*	OIE (2006a,b)
Black sea bream	*Acanthopagrus schlegelii*	Isshiki *et al*. (2003)
Blue whiting	*Micromesistius poutassou*	OIE (2000)
Bluegill	*Lepomis macrochirus*	OIE (2006a)
Brook trout	*Salvelinus fontinalis*	Rasmussen (1965)
Brown trout	*Salmo trutta*	Rasmussen (1965)
Chinook salmon	*Oncorhynchus tschawytscha*	Winton *et al*. (1989)
Coho salmon	*Oncorhynchus kisutch*	Winton *et al*. (1989)
Dab	*Limanda limanda*	OIE (2000)
English sole	*Parophrys vetulus*	Hershberger *et al*. (1999)
Eulachon	*Thaleichthys pacificus*	Kaufman and Holt (2001)
European eel	*Anguilla anguilla*	Castric *et al*. (1992)
Flounder	*Platichthys flesus*	OIE (2000)
Freshwater drum	*Aplodinotus grunniens*	OIE (2006a)
Gizzard shad	*Dorosoma cepedianum*	OIE (2006b)
Golden trout	*Salmo aguabonita*	Ahne *et al*. (1976)
Grayling	*Thymallus thymallus*	Meier and Wahli (1988)
Greenland halibut	*Rheinhardtius hippoglossoides*	Dopazo *et al*. (2002)
Haddock	*Melanogrammus aeglefinus*	Smail (2000)
Japanese amberjack	*Seriola quinqueradiata*	Ito *et al*. (2004)
Japanese flounder	*Paralichthys flesus*	Takano *et al*. (2000)
Lake trout	*Salvelinus namaycush*	Dorson *et al*. (1991)
Lamprey (sea)	*Petromyzon marinus*	OIE (2004)
Largemouth bass	*Micropterus salmoides*	OIE (2003a)
Lesser argentine	*Argentina sphyraena*	OIE (2000)
Mummichog	*Fundulus heteroclitus*	Gagne *et al*. (2007)
Muskellunge	*Esox masquinongy*	OIE (2006a)
Norway pout	*Trisopterus esmarkii*	Mortensen *et al*. (1999)
Pacific cod	*Gadus macrocephalus*	Meyers *et al*. (1992)
Pacific hake	*Merlucius productus*	Meyers *et al*. (1999)
Pacific herring	*Clupea pallasi*	Meyers *et al*. (1994)
Pacific mackerel	*Scomber japonicus*	Cox and Hedrick (2001)
Pacific sand eel	*Ammodytes personatus*	Watanabe *et al*. (2002)
Pacific sand lance	*Ammodytes hexapterus*	Kocan *et al*. (2001)
Pike	*Esox lucius*	Meier and Jorgensen (1979)
Pilchard	*Sardinops sagax*	Traxler *et al*. (1999)
Plaice	*Pleuronectes platessa*	OIE (2000)
Poor cod	*Trisopterus minutus*	King *et al*. (2001)
Rainbow trout	*Oncorhynchus mykiss*	Jensen (1963)
Red sea bream	*Pagrus major*	Ito *et al*. (2004)
Red-spotted grouper	*Epinephalus akaara*	Isshiki *et al*. (2003)
Rockfish	*Sebastes inermis*	Isshiki *et al*. (2003)
Rockling, four-bearded	*Rhinonemus cimbrius*	OIE (2000)
Round goby	*Neogobius melanostomus*	OIE (2006b)
Sablefish	*Anoplopoma fimbria*	OIE (1999)
Sand eel	*Ammodytes* sp.	Skall *et al*. (2005)

(*Continued*)

Table 3.1. *Continued*

Host Species	Specific name	Reference
Sand goby	*Pomatoschistus minutus*	Skall *et al.* (2002)
Sand lance	*Ammodytes personatus*	Watanabe *et al.* (2002)
Sardine	*Sardinops sagax*	Hedrick *et al.* (2003)
Schlegel's black rockfish	*Sebastes schlegelii*	Isshiki *et al.* (2003)
Sea bass	*Dicentrachus labrax*	Castric and de Kinkelin (1984)
Shiner perch	*Cymatogaster aggregata*	Kent *et al.* (1998)
Shorthead redhorse	*Moxostoma macrolepidotum*	OIE (2006b)
Silver redhorse	*Moxostoma anisurum*	OIE (2006b)
Smallmouth bass	*Micropterus delomieui*	OIE (2006a)
Smelt	*Thaleichthys pacificus*	Hedrick *et al.* (2003)
Sprat	*Sprattus sprattus*	OIE (2000)
Steelhead trout	*Oncorhynchus mykiss*	Brunson *et al.* (1989)
Striped bass	*Morone saxatalis*	OIE (2003b)
Surf smelt	*Hypomesus pretiosus*	Hedrick *et al.* (2003)
Tomcod	*Microgadus proximus*	Meyers *et al.* (1999)
Three-spined stickleback	*Gasterosteus aculeatus*	Kent *et al.* (1998)
Turbot	*Psetta maxima*	Schlotfeldt *et al.* (1991)
Yellow perch	*Perca flavescens*	OIE (2006b)

whitefish (*Coregonus* sp.) (Reichenbach-Klinke, 1959; Ahne and Thomsen, 1985; Meier *et al.*, 1994).

While Atlantic salmon is susceptible to experimental infection by intraperitoneal infection (de Kinkelin and Castric, 1982), an important distinction must be made between natural hosts of VHS and experimental hosts. Atlantic salmon in European coastal waters is not considered a natural host of VHS virus and, to date, represents only an experimental model. However, only time will tell whether any marine strains of VHSV can be pathogenic to Atlantic salmon.

Farmed marine species, such as sea bass (*Dicentrarchus labrax*) and turbot from the Mediterranean area, are susceptible. This was shown experimentally by Castric and de Kinkelin (1984). Virus multiplication was demonstrated in both species and histopathology described. Clinical disease in VHS outbreaks on turbot farms has also been described in Germany by Schlotfeldt *et al.* (1991) and in Scotland on Gigha Island by Ross *et al.* (1994). In the latter case, it was the largest tank-reared, 3-year-old turbot that showed the highest mortality, suggesting that the infection had been dormant for some months during growth.

Looking to the wider distribution of VHSV and its host range, a significant finding was the discovery of virus in anadromous salmonids in 1988 off the western Pacific coast of the USA, i.e. in migrating coho salmon (*O. kisutch*) and Chinook salmon (*O. tshawytscha*). The first Pacific-type strain of VHSV, termed Makah, was identified and named after the Makah National Fish Hatchery in Washington State, USA, where the migratory fish were sampled (Brunson *et al.*, 1989). The isolation of this first Pacific strain of virus was unexpected and led regulatory authorities to ask whether VHSV had a marine source and a global distribution; also whether the marine Pacific strains were different in genetic constitution to the established European strains from freshwater trout.

In 2009, the disease was still seen in Continental Europe in trout farms. In the mid-1990s, VHS was confirmed in turbot at a marine fish farm in Scotland, UK (Ross *et al.*, 1994), and Ireland in 1996 (J. McArdle, personal communication). Marine forms of the virus are known from Alaska and Washington State, USA, in the Pacific Ocean. Marine forms of the virus have also been isolated from the North Sea in cod with skin

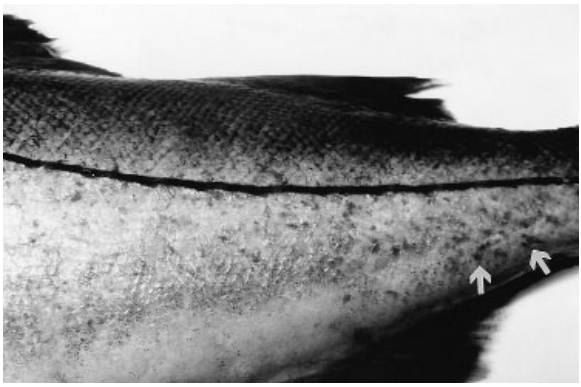


Fig. 3.1. Haddock (*Melanogrammus aeglefinus*) tail showing skin erythema (arrowed) from which VHS virus was isolated, east of Shetland, Scotland, June 1995.

ulcers and haddock with erythema (Fig. 3.1) (Smail, 2000).

The global distribution of the virus in 2009 included the western Pacific coastline of the North American continent, the Great Lakes, the eastern seaboard of Canada, the Flemish cap area of the western Atlantic Ocean, the coastal seas around the UK, the Baltic Sea, Skaggerak and Kattegat, the continental waters of inland Europe, the eastern coastal waters of the Black Sea off northern Turkey, the Pacific Ocean and the south Japanese coast. This global map highlights that the distribution of VHSV occurs between latitudes of 60° and 30° north, which may correlate with temperature minima and maxima for replication of the virus.

VHSV has not been found to date in the southern hemisphere where salmonid aquaculture is practised, i.e. Australia, Chile, New Zealand and South Africa (Skall *et al.*, 2005).

Economic Importance of the Disease

The accurate current cost of VHS outbreaks is not often quoted in the literature, nor is it easy to access. Skall *et al.* (2005) quoted the estimate of Hill (1992) that losses due to VHS in salmonid European aquaculture amounted to £40 million/year in 1991. However, the current inflation-adjusted cost for the European industry against the stock investment is not reported and there is a need for accurate economic reports for government and academia. Skall *et al.* (2005) also quoted the study of Nylin and Olesen (2001), where the cost of VHS outbreaks at two farms with mortalities of around 50% was estimated at above €211,000.

Diagnostic Methods

Clinical diagnosis

Many of the clinical signs for diseased rainbow trout are described above. The key external signs include pale gills, accumulation of ascitic fluid or ascites leading to a swollen abdomen, iris eye haemorrhage and exophthalmia in one or both eyes and faecal casts which may be clear, yellow or blood tinged. In free-swimming fish, behavioural signs may be seen in the late nervous form of VHS, including flashing, leaping or spiralling due to inflammation and irritation of infected nervous tissue.

Culture

A variety of established fish cell lines are susceptible to VHSV. Bluegill fry (BF-2) and rainbow trout gonad (RTG-2) cells are the most sensitive cell lines for a serogroup I freshwater virulent isolate from rainbow trout and also a serogroup III isolate (Olesen

and Vestergård-Jørgensen, 1992; Lorenzen, E. *et al.*, 1999). Other possible cell lines for use are rainbow trout gonad (RTG-2), *epithelioma papulosum cyprini* (EPC), fathead minnow (FHM), Chinook salmon embryo (CHSE-214) or pike gonad (PG) (Wolf, 1988). Cell lineage and passage number can have a marked effect on virus replication, as evidenced by titres of several fish viruses including VHSV on CHSE-214 cells (McAllister, 1997). The cytopathic effect (CPE) consists of pronounced cell shrinkage, with some cell rounding. In RTG-2 cells, a partial differential diagnosis of virus type can be made depending on the plaque type; VHSV causes 'staggered edge' plaques in RTG-2 cells, whereas IPNV causes more even-edged plaques.

Immunodiagnosis

Virus confirmation

Once viral CPE is recognized, the supernatant virus is harvested and the virus identified using one of several techniques. The virus can be identified using a serum neutralization test on any susceptible cell lines. This is carried out using either a plaque-neutralization test (PNT) or a microtitre neutralization test, using a constant dose virus/varying antiserum dilution format.

The PNT for VHSV confirmation is highly dependent on heat-sensitive serum factors in normal serum, i.e. complement. These normal serum proteins assist and stabilize the antigen–antibody cross-linking bonds.

IMMUNOFLUORESCENT ANTIBODY TEST (IFAT). An IFAT was developed by Vestergård-Jørgensen and Meyling (1972) to detect viral antigen in infected tissue cells up to 26 h post-infection. Maximum staining was detected from 18 to 26 h at peak protein synthesis and virus assembly. Staining works well with either a cross-reacting specific MAb to the virus or a polyclonal antibody. A secondary antibody labelled with fluorescein is used which tags the primary antibody and labels infected cells, which fluoresce under blue light.

The technique of immunofluorescent staining for a viral antigen and viral antibody in the same tissue preparation was perfected by Vestergård-Jørgensen (1974) with clarity and brilliance. Antibody detection using IFAT involves fixed cells with viral antigen stained with test sera, and then an antispecies antibody labelled with fluorescein is added. Positive slides will show foci of fluorescence positivity, compared with appropriate negative and positive controls.

ENZYME-LINKED IMMUNOSORBENT ASSAY. The antigen-capture enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of virus in culture supernatants. The first layer in the ELISA is the catching antibody, normally a polyclonal antibody to VHSV, but alternatively this may be a MAb. The second step is a blocking reaction to coat unadsorbed solid-phase sites, typically with bovine serum albumin or milk protein, and the third step is the addition of virus. The fourth step is the addition of MAb, normally to G protein, and the fifth is the addition of an antispecies antibody to MAb, which is tagged with an enzyme. Way and Dixon (1988) described VHSV ELISA for a polyclonal direct antigen-capture system. Mourton *et al.* (1990) reported on three modes of ELISA for virus detection: indirect ELISA, direct ELISA and antigen-capture ELISA, using a variety of MAbs to the viral glycoprotein G. This resulted in virus detection at a protein concentration as low as 1 ng/ml of total proteins where 1.5 fmol/ml of envelope glycoprotein was present.

Antibody detection

Detection of fish antibodies is a good indicator of previous infection; however, to date, serology has not been accepted as a routine diagnostic method for assessing the virus status of fish populations (Lorenzen and LaPatra, 1999). Such antibodies can be detected using ELISA, IFAT or a PNT, as described by Olesen *et al.* (1991). At that time, ELISA was reported as the most sensitive technique, and different types of antibody were clearly involved,

complement-dependent for neutralization and non-complement dependent for ELISA. For example, high values of antibody by one assay may be contrasted with low values by another; such as, ELISA-positive and PNT-negative in one fish and ELISA-negative and PNT-positive in another. Fregeneda-Grandes and Olesen (2007) compared ELISA, 50% PNT and Western blotting (WB) for antibody detection in an enzootic VHSV infected population of 50 rainbow trout. The 50% PNT was shown to demonstrate the highest sensitivity (90%) versus 41% of samples for the ELISA when tested against the local virus strain, and 61% for the WB. The authors commented, 'the variable specificity of the ELISA test makes the neutralization test the technique of choice for the screening of non-infected populations where false-positive reactions could have damaging consequences'. The most important point was that the choice of virus strain was crucial: sera showed no neutralization against DK-F25 and F1 type I neutralization pattern isolates and 90% against the local type III pattern isolate.

PCR and real-time PCR

A number of PCR methodologies have been reported as suitable for detecting the presence of VHSV RNA which are based on similar principles. RNA is first reverse transcribed into complementary DNA (cDNA) using a suitable primer, followed by amplification of this cDNA using PCR with a primer set designed to bind specifically to conserved areas within the VHSV genome. The resultant amplification product is visualized under UV light following electrophoresis in agarose gels and staining with ethidium bromide. The method reported by Snow *et al.* (2004) has proven capable of amplification of a wide range of VHSV genotypes and is currently recommended for use by the current version (6th edition, 2009) of the OIE *Manual of Diagnostic Tests for Aquatic Animals* (http://www.oie.int/eng/normes/fmanual/A_summry.htm). López-Vázquez *et al.* (2006) developed a rapid, sensitive, non-lethal diagnostic nested RT-PCR for the detection of VHSV from blood samples of experimentally infected brown trout. The nested PCR detected positives in 85% of fish versus 40% for RT-PCR and southern blot, with only 5% of samples resulting in virus isolation. These sensitivities may reflect a low level viraemia in the blood of brown trout.

Increasingly, molecular diagnosticians are using real-time PCR for VHSV diagnostics. Real-time or quantitative PCR (qPCR) is based on the principle of conventional PCR, and a number of alternative methodologies exist (for a review, see Bustin *et al.*, 2005). Both SYBR green and Taqman™ assays have been reported in the literature for the detection of VHSV (Chico *et al.*, 2006; Matejusova *et al.*, 2008; Cutrin *et al.*, 2009). SYBR green is a DNA binding dye and, during formation of new PCR amplicons, increasing amounts of dye bind to the nascent double-stranded DNA. The increase in fluorescent dye is measured in 'real time', allowing the user to monitor amplification as it progresses. Taqman™-based methods are founded on a similar principle, but a VHSV sequence-specific, fluorescently labelled probe is bound to single-stranded DNA targets in the PCR reaction. The 5′ nuclease activity of the polymerase cleaves the labelled probe during amplification, resulting in the release of a fluorescent dye, which is measured in real time and is proportional to the amount of amplification. Taqman™-based probes offer a second level of specificity over other methods since they rely on both initial amplification using a PCR primer set, in addition to specific binding of a secondary probe to the primary amplification product. Probe selection in such assays must be targeted to highly conserved gene regions since the disadvantage of such specificity is that a single nucleotide substitution in the probe region can lead to a negative result. Matejusova *et al.* (2008) developed a rapid, accurate and sensitive quantitative real-time RT-PCR (qRT-PCR) that targeted a conserved region of the N gene of the virus. It was demonstrated to be more sensitive than the conventional RT-PCR and detected all three European genotypes I, II and III.

In addition to being very specific, real-time PCR is also sensitive due to the use of small amplicon targets coupled to the detection chemistries employed. Real-time PCR is quicker than conventional PCR since no electrophoresis step is required, and the method also lends itself well to high-throughput automation. The method is also suited to the inclusion of positive and endogenous controls present in all tissue types to check template quality, as reported for VHSV. Results can also be interpreted quantitatively, giving some idea of the level of target RNA present in a sample. This factor is exploited in studies of gene expression.

Nucleotide sequencing of conventional PCR products remains the best way of confirming the detection of VHSV, in addition to obtaining the greatest amount of information regarding the molecular epidemiology of VHSV. This can facilitate the rapid genetic typing of new isolates. Other more rapid methods have been reported, however, that can differentiate between genetic groups of VHSV isolates based either on PCR alone or on PCR followed by restriction fragment length polymorphisms (RFLPs) (Einer-Jensen *et al.*, 2005).

Recently, a loop-mediated isothermal amplification (LAMP) detection method has been described which offers potential for simplifying detection of VHSV (Soliman and El-Matbouli, 2006).

Genome Structure and Transcription

A number of full genome sequences for VHSV have been obtained. As with other members of the order *Mononegavirales*, the VHSV genome consists of a single-stranded, non-segmented negative strand RNA molecule of approximately 12,000 nucleotides. The genome is organized as follows: 3-N-P-M-G-NV-L-5′ (Basurco and Benmansour, 1995). The synthesis of rhabdovirus messages is generally preceded by the transcription of a short (+)-sense leader RNA that presumably is the first viral product made in an infected cell (Colonno and Banerjee, 1977). In the case of VSV and RV, transcription of the genome generates a short, untranslated leader RNA of 47 and 58 nucleotides, respectively, followed by monocistronic mRNAs that encode the viral proteins. In contrast, the leader sequence of the fish lyssaviruses is found even on clones obtained by reverse transcription of messenger RNA (Gilmore and Leong, 1988; Bernard *et al.*, 1990). A number of full genome coding sequences for VHSV are now available (Campbell *et al.*, 2009).

Viral gene/protein structure

Nucleoprotein gene

The N gene European VHSV isolates consist of an open reading frame (ORF) of 1212 nucleotides (isolate 07-71), though an additional stretch of residues has been reported at the 3′ end of the coding region in North American isolates (Batts *et al.*, 1993). The sequencing of clones prepared from polyadenylated RNA has revealed that the N gene open reading frame is preceded by a leader sequence of around 93 bp, including the transcription start signal (AACA) (Bernard *et al.*, 1990, 1992). The nucleoprotein is bound to the viral RNA and plays an important role in the transcription and replication processes (Bernard *et al.*, 1992). Using a 'north-western technique', Said *et al.* (1995) have demonstrated that amino acids (aa) 145–254 interact specifically with the genomic or messenger RNA of VHSV.

Phospho (P) and matrix (M) protein genes

The P gene and M gene ORFs are around 666 and 603 nt, respectively. The matrix proteins of rhabdoviruses are highly basic and are thought to play an important role in virion assembly and maturation (Benmansour *et al.*, 1994). Both the P and M proteins of VHSV share several features which are similar to those described for equivalent proteins of VSV and RV. The polymerase-associated proteins of VSV, RV and VHSV are all phosphorylated. Phosphorylation of the phosphoprotein (P) of VSV, first by a cellular kinase and then by the viral L protein, is essential for transcription of the N

RNA template by the RNA polymerase complex (Barik and Banerjee, 1992). The M protein of VHSV is highly basic and binds to the N protein and presents a positively charged terminal domain, which in VSV is implicated in transcription inhibition and in binding the nucleocapsid. Thus, it is likely that they exert similar functions (Benmansour *et al.*, 1994).

Glycoprotein gene

The VHSV G gene is 1521 nucleotides long, encoding a polypeptide of 507 amino acids and with an Mr of 65 kDa, which is reduced to 55 kDa by deglycosylation (Thiry *et al.*, 1990). Comparison of VHSV and IHNV G proteins revealed a 38% identity with conserved features, indicating structural and functional homologies beyond general rhabdoviral similarities (Lorenzen *et al.*, 1993). The occurrence of 15 out of 16 cysteine residues in identical positions indicates strongly that the three-dimensional structures of the two viral glycoproteins are very similar (Lorenzen *et al.*, 1993). Cysteine residues are important in preserving the secondary and tertiary structure of proteins due to their ability to form intramolecular disulfide bonds (Matsumura *et al.*, 1989).

The homotrimeric membrane glycoprotein is responsible for the attachment of virus to the cellular membrane and contains the low pH-dependent membrane fusion activity present in both mammalian (Gaudin *et al.*, 1992, 1993) and fish (Lecocq-Xhonneux *et al.*, 1994) rhabdoviruses. The binding of synthetic peptides to phosphatidylserine (PS) has identified the major phospholipid binding domain to be located between amino acids 82–109 of the VHSV glycoprotein (designated p2), and subsequent comparative sequence analysis identified that p2-like sequences exist in all rhabdoviruses (Coll, 1995b).

The glycoprotein is also thought to play an important role in the induction of protective anti-VHSV immunity *in vivo*, as neutralizing antibodies (NAbs) to VHSV exhibit exclusive specificity for this protein (Bernard *et al.*, 1983; Lorenzen *et al.*, 1990; Estepa *et al.*, 1994). NAbs are one of the immunological factors believed to constitute an important component of the protective immune response against VHSV (Lorenzen *et al.*, 1990) and against rhabdoviruses in general (Kelley *et al.*, 1972; Celis, 1990). In addition, the glycoprotein has been demonstrated to stimulate trout leucocytes from uninfected fish (Estepa and Coll, 1991), VHSV immunized fish (Estepa and Coll, 1991) and survivors of VHSV infection (Estepa and Coll, 1992). Neutralizing antibody-resistant mutants have been widely used to map the epitopes involved in the neutralization of many rhabdoviruses. Most neutralizing epitopes of rhabdoviruses, including VSV (Luo *et al.*, 1988), RV (Benmansour *et al.*, 1991) and IHNV (Huang *et al.*, 1996), have been located within the central portion of the viral glycoprotein (Jørgensen *et al.*, 1995).

It is also known that the glycoprotein gene can play a role in the virulence of VHSV (Béarzotti *et al.*, 1995c). Based on the selection of NAb escape mutants with reduced pathogenicity for fish, Béarzotti *et al.* (1995c) identified a region formed by the association of amino acids at positions 140 and 433 that appeared to play an important role in the determination of the virulent status.

Non-virion protein gene

Sequence analyses of the genomes of 18 different VHSV strains representing the four serotypes isolated from Europe and the USA have revealed the presence of an NV gene at the G–L junction, as has been described previously for IHNV (Basurco and Benmansour, 1995). A similar gene was also identified in hirame virus (HIRV), a virus thought to be related closely to IHNV, but was not present in the vesiculo-like fish rhabdovirus, SVCV (Kurath *et al.*, 1997). Since there are no reports of genes analogous to NV in the genomes of viruses infecting non-fish hosts, the NV gene may be a characteristic feature of fish lyssaviruses (Kurath *et al.*, 1997). Comparison of the NV sequences available for VHSV isolates (Morzunov *et al.*, 1995; Schuetze *et al.*, 1995; Kurath *et al.*, 1997) has revealed a

high divergence between strains from the two continents, but a remarkable degree of sequence conservation among strains originating from the same continent, regardless of their serotype (Basurco and Benmansour, 1995).

The NV protein of VHSV consists of 122 aa, with a calculated Mr of 13.6 kDa. While the NV proteins of IHNV and HIRV have distinct similarities in both amino acid sequence and overall structure, the NV protein of VHSV is very different (Kurath *et al.*, 1997). Comparisons of amino acids have further revealed that the NV genes of IHNV, HIRV and VHSV are significantly more diverse than the other structural proteins of the same three viruses (Basurco and Benmansour, 1995; Morzunov *et al.*, 1995; Kurath *et al.*, 1997). This suggests that there is a relatively low level of evolutionary constraint on the NV gene, although the exact function of the NV protein remains unclear.

Polymerase protein gene

To date, the VHSV polymerase gene has not been characterized extensively, although sequence data exist for the related rhabdoviruses, IHNV and SVCV (Björklund *et al.*, 1995). Preliminary comparisons of L gene sequence have indicated that while SVCV is related closely to mammalian vesiculoviruses, IHNV may be related only distantly to mammalian lyssa and vesiculotype rhabdoviruses (Björklund *et al.*, 1995).

The L protein is, through an interaction with the phosphoprotein and nucleocapsid protein, responsible for the RNA-dependent RNA polymerase enzyme activities involved in virus transcription and replication.

Pathogenesis and Immunity

The host–pathogen interaction is finely balanced in the case of VHS virus. Predisposing factors may induce a disease state when there is sufficient initial virus to replicate in the target tissues. Thereafter, the course of disease as to recovery or lethality is influenced by the potency and efficacy of the innate and humoral immune responses to limit virus replication and the extent of internal bleeding the virus causes. Studies in the decade 2000–2009 have contributed greatly to our understanding of the cytokine responses against VHSV, especially the efficacy of host interfering Mx proteins to limit infection (see references under the section below on innate immunity).

Predisposing factors

Stress

In common with most fish viral diseases, stress plays a role in the induction of disease, inducing recurrence or recrudescence of the infection from a carrier state. An example of this was described by Horlyck *et al.* (1984) in marine-cultured rainbow trout in Denmark. Overt disease was seen in rainbow trout growers in seawater that previously has been screened virus negative in fresh water, and mortality varied from 12 to 50% in four cases. A possible explanation could have been that there was a silent VHS infection in the fingerling and grower trout in freshwater trout, escaping standard tissue-culture detection, which was reactivated later after the stress of handling or difficulty with osmoregulation in seawater. *De novo* induction of VHS as an apparent recurrence of disease could point to a modulating role for the stress hormone cortisol and other corticosteroids in the induction of VHSV replication in the kidney and spleen, the principal leucopoietic tissues. However, an alternative explanation for the new isolation in seawater-cultured rainbow trout could have been *de novo* infection from a marine source.

Age

Rainbow trout fry of 0.3–3.0 g are the most susceptible to disease, with target organs that represent most of the body weight. Mortality of 80–100% due to virulent strains is normal at optimum temperatures of 9–12°C. Fingerlings and growers are also susceptible but mortality is lower, in the range of 10–50% for virulent strains, with

the nervous chronic state more commonly seen in these fish.

Temperature

Viral haemorrhagic septicaemia is a cold-water disease, with a temperature range of 2–12°C, although the optimum is 9–12°C for rainbow trout, where replication of the virus is greatest. Temperatures above 15°C are inhibitory to viral growth. Vestergård-Jørgensen (1982) showed that temperature was correlated inversely with the longevity of the infection in rainbow trout. In groups of 11 g trout transferred to 5, 10, 15 or 20°C after infection at 10°C, the infection was persistent for 14 weeks at 5°C, in contrast to 2 weeks at 10°C and undetectable at 20°C. Neukirch (1985) showed that the infection persisted for 300–400 days in rainbow trout at 4°C.

Signs and pathological changes

A common feature in all hosts, salmonids or flatfish, is the widespread haemorrhaging seen internally, over the liver, in adipose tissue (Fig. 3.2) and especially within the muscle.

External signs

Affected rainbow trout fry are lethargic and darker in pigmentation, swim erratically or have difficulty in orientation and may congregate along the sides of tank and pond outlets. Flashing, corkscrewing motion and surface swimming are also seen in the later nervous-form-affected trout fingerlings. Exophthalmia may be seen in one or both eyes (Figs 3.3 and 3.4) and there can be haemorrhage around the eye orbit (Fig. 3.5).



Fig. 3.2. Rainbow trout (*Oncorhynchus mykiss*) fingerling with clinical VHS, showing oedema, pale liver and haemorrhage over the adipose tissue (arrowed).



Fig. 3.3. Rainbow trout (*Oncorhynchus mykiss*) fingerling with clinical VHS, showing exophthalmia and pale necrotic kidney (arrowed).



Fig. 3.4. Turbot (*Psetta maxima*) with VHS at a farm outbreak, showing exophthalmia.

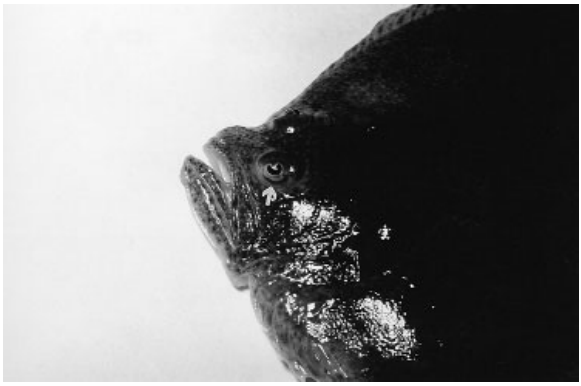


Fig. 3.5. Turbot (*Psetta maxima*) experimental VHS infection with a virulent freshwater strain, showing haemorrhaging around the eye orbit (arrowed).

The gills are markedly pale, reflecting generalized anaemia.

Internal signs

The most pronounced changes are in the kidney and liver. The kidney is swollen and darker red in the early stages, but the head and midsection are often totally necrotic and pale in dead trout fry and fingerlings (see Fig. 3.3). The liver is paler than normal, yellowish, with areas of mottled haemorrhage. A systemic haemorrhage may be evident in the ocular tissues and skin and in the viscera, including the intestinal submucosa and skeletal musculature. There is no food in the gastrointestinal tract, with stringy mucus in the lumen.

Histopathology

Viral haemorrhagic septicaemia virus is most pathogenic to the kidney (Fig. 3.6), and early necrosis of the haematopoietic tissue rather than the excretory tubules is a marked feature of the disease. The melanomacrophages, distributed at high density throughout the head kidney, lyse with release of granules and necrose. VHSV has a marked leucotropism, as shown by the fact that mitogen-stimulated leucocytes are lysed by VHSV in *in vitro* culture (Estepa and Coll, 1991). In contrast, the birnavirus infectious pancreatic necrosis virus (IPNV) sets up a persistent or non-lytic infection (Knott and Munro, 1986).

In rainbow trout, the liver shows widespread necrotic degeneration of hepatocyte nuclei, granulation of chromatin and the accumulation of hyaline material in the tubular lumen. An example of liver pathology in experimentally infected turbot is illustrated in Fig. 3.7. In other hosts, especially pike fry, there may be extravasation or bloody swelling, deposition of blood in the muscle and pancreatic necrosis (Meier and Vestergård-Jørgensen, 1980). The liver sinusoids become engorged with blood and show widespread necrosis and many pyknotic and karyolytic nuclei without

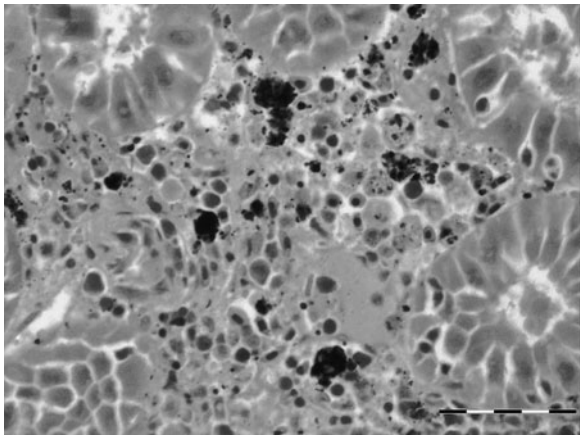


Fig. 3.6. VHS in rainbow trout (*Oncorhynchus mykiss*), experimental infection due to strain 07-71, kidney section. Note the degranulated macrophages in the haematopoietic tissue at the centre of the field. H & E. Scale bar represents 20μm.

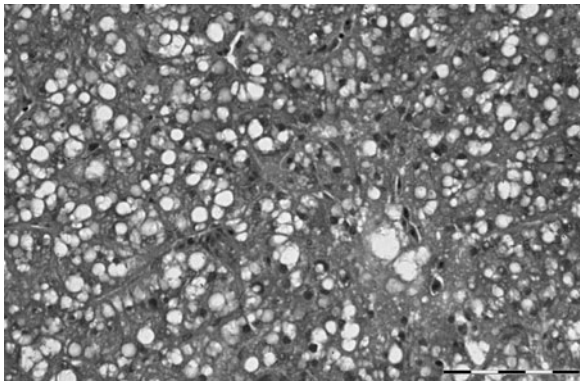


Fig. 3.7. VHS in turbot (*Psetta maxima*), experimental infection, strain 07-71, liver pathology showing widespread necrosis and vacuolation of hepatocytes. H & E. Scale bar represents 20μm.

other diagnostic inclusions. Many foci of erythrocytes occur within the musculature.

The heart is also a target organ. While heart pathology has not been described in salmonids, a very marked necrosis of the ventricular fibres was seen in turbot from the VHS outbreak at Gigha Island, Scotland (Ross *et al.*, 1994). Heart failure is a probable prime cause of death in this bottom-living species, as the rate of opercular movement increases in moribund VHSV infected turbot.

Varied forms of the disease

In rainbow trout, the disease occurs in three varied forms, i.e. acute, chronic and nervous, followed by a carrier state in surviving fish, where the virus can be isolated from persistently infected tissues, such as kidney and brain.

In the acute stage (experimental), from day 0 to 30 at 8–12°C, sick fish lie on the bottom of the tank or are moribund, displaying the preacute signs of flashing or corkscrewing. The chronic stage reflects a lasting persistent infection and is correlated with no obvious external signs. Virus can be isolated from all the major internal organs and there are subacute depressive effects of the virus on red and white blood cell counts. In the nervous form, there are very marked aberrations of swimming behaviour, i.e. constant flashing, tail-chasing and spiralling. This is associated with a most marked tropism of the virus to the brain, and brain virus titres of 1×10^9TCID50/g can be attained for the 07-71 strain in nervous-form

rainbow trout by experimental challenge (Smail, unpublished observations).

Nervous tropism is a feature of virulent freshwater strains, such as type F1 Voldbjerg or 07-71, but can be observed with marine strains, such as that from turbot. The nervous tropism of the virulent strains is not understood completely but is likely to be related closely to the specific interaction of the glycoproteins in the virus envelope with membrane lipoproteins in target cells of susceptible nervous tissue. In this connection, Béarzotti *et al.* (1995a) have identified two loci for amino acid substitution in the glycoprotein gene (residues 140 and 430), which are associated uniquely with reduced virulence and increased tissue tropism for nervous tissue.

Disease progression

Viral haemorrhagic septicaemia virus gains entry to fish by crossing the gill epithelium and thence, via the blood, to the main internal organs. There is also evidence that the skin can be a portal of viral entry. Yamamoto *et al.* (1992) showed that skin explants could be infected with virus, with increasing titres in time. Quillet *et al.* (2007a) showed that, by using an assay for viral replication in excised fin tissue (VREFT) (Dorson and Torhy, 1993), the degree of VHSV replication in fin tissue of waterborne challenged rainbow trout was correlated positively with the mortality level in waterborne VHSV challenge of the same species. Therefore, the VREFT assay was a good indicator of host susceptibility. It is possible that VHSV infected skin cells are also a portal of exit for the virus, but there is no evidence to support or disprove this. Investigations indicate that polarized gill cells at the base of the fins are particularly important in the uptake and entry of not only VHSV by bath infection (Brudeseth *et al.*, 2008) but also IHNV (Harmache *et al.*, 2006).

By adhering to, penetrating and multiplying in the endothelial cells lining venules and sinusoids, the virus causes the first degree of pathology to cells lining the circulatory system from 48 h after infection. Then, the virus spreads to the head kidney and causes cell degeneration and necrosis of haematopoietic cells of the kidney and of macrophages and melanomacrophages from 2–4 days post-infection. Next, infection spreads to the liver (4 days), with necrosis of hepatocytes, and, later, necrosis of pancreatic acinar cells (6 days) (Evensen *et al.*, 1994). It was shown by Evensen *et al.* (1994) for the Fl strain infection of rainbow trout that immunohistochemical staining for viral antigen could demonstrate virus distribution. However, at day 2 post-infection, virus could be isolated without positive immunostaining, showing virus isolation to be more sensitive than immunostaining.

Shedding of virus from carrier fish takes place via the urine by day 3 post-infection in experimental bath infection of rainbow trout (Neukirch, 1985), but there is no evidence of faecal excretion, since the virus is sensitive to gut acids. It is possible that in marine fish, such as cod and haddock (see Fig. 3.1), the skin is also a source of viral shedding.

In marine species, e.g. turbot, a similar pattern of pathogenesis takes place, with haemorrhaging over the eye orbit and fin margins and marked swelling, due to impaired osmoregulation (see Figs 3.4 and 3.5). The sequence of virus entry, via gills to bloodstream and thence to the target organs, head kidney and liver, is also repeated. Necrosis of target haematopoietic cells quickly leads to kidney malfunction and morbidity (Castric and de Kinkelin, 1984).

Disease mechanisms/pathophysiology

The kidney, heart and liver are target organs for the virus. Overt cell and tissue necrosis is seen in these tissues, which, in the case of the kidney, leads quickly to a loss of tubular excretory function and correct osmoregulation – hence, the commonly observed oedema and bloated appearance. Liver necrosis will lead to loss of glycogen and energy reserves and heart function will be

impaired at slight levels of myocardial cell necrosis. The hallmark of VHSV infection of rainbow trout is haemorrhaging, for example, around the eye, within the visceral adipose tissue or the body musculature. These external and internal signs of disease reflect the susceptibility of the ultrafine blood capillaries, blood venules and endothelial cells of the circulatory system to the virus, so much so that VHSV budding from these cells actually breaks the fine circulation with the release of erythrocytes into the tissues. This is most obvious in the muscle and around the eye orbit.

Characterization of virulence factors

Specific virulence factors for VHSV are not well understood but are likely to be multifactorial and are certainly species specific. Interestingly, Brudeseth *et al.* (2008) reported virulence in rainbow trout to be determined by the ability of VHSV to translocate primary cultures of gill epithelial cells. Quillet *et al.* (2007a) earlier demonstrated a correlation between replication of VHSV in fin tissues and the mortality in subsequent challenge. Harmache *et al.* (2006) demonstrated the importance of cells at fin bases for the initial replication of the related novirhabdovirus, IHNV. No doubt the ability to infect initial target cells efficiently is of fundamental importance to the outcome of viral infection. Following initial infection, the outcome of viral pathogenesis is likely a result of the relative ability of the virus to circumvent the array of host cellular defences, some of which are discussed below. Examples of new emergence of disease in rainbow trout reared in seawater (Raja-Halli *et al.*, 2006), coupled with experimental evidence that most naturally occurring marine viruses are of low virulence to rainbow trout (Skall *et al.*, 2004), suggests that the virulence characteristics of VHSV can be subject to change under the selective regime of intensive aquaculture. Sequencing the full genome of two related VHSV strains of demonstrated differing virulence recently identified only five amino acid substitutions (Campbell *et al.*, 2009). While the specific effect of these changes is yet to be demonstrated, this study does confirm that very few changes in the genome of VHSV are required to confer a change in virulence properties. It is likely that the development of reverse genetic approaches, coupled with increased availability of sequence information, will lead to a greater understanding of the relevance of small changes in different genes in determining virulence.

Innate immunity

Innate immunity towards VHSV is of two types; the type I (α/β) interferon (IFN)-inducible Mx proteins, which are highly conserved GTPases (Haller *et al.*, 1998), and antiviral cytotoxic cells which are able to lyse virus-infected cells and thus reduce the rate of virus multiplication. These have been defined as the responsive innate mechanism (IFN-Mx) and the constitutive response (non-specific cytotoxic cells) (Ellis, 2001). The latter cellular innate mechanisms have been little studied by contrast to IFN, although first results are emerging on how crucial the early innate cellular immune response is for determining host susceptibility of carrier or reservoir fish species for VHSV.

Interferon and Mx proteins

Ellis (2001) has reviewed the IFN and Mx protein response against viral diseases and emphasized that as viral double-stranded RNA (ds-RNA) is a potent inducer of IFN, in the case of VHSV, the replicative intermediate ds-RNA will stimulate IFN as soon as it is produced. Therefore, in evolutionary terms, the ability to recognize these molecules as foreign and respond to them by this innate mechanism has had a selective advantage. Robertsen (2008) reviewed expression of IFN and interferon-induced genes (ISGs) in salmonids in response to virus infection, IFN-inducing compounds and vaccination. In particular, the expression of ISGs in response to VHSV infection was reviewed here. An

earlier review covered the IFN system of teleost fish (Robertsen, 2006).

The techniques and methodologies for interferon detection have advanced in recent years, with results published on the kinetics of Mx expression in salmonids. Collet *et al.* (2004) originated a cell line, RTG-P1, transfected permanently with the luciferase gene under control of the trout Mx promoter which was induced by IFN. Cells responded to poly I:C, a double-stranded RNA molecule and an IFN stimulant, by releasing IFN into the medium, which autostimulated the reporter to produce luciferase. In a related study, Acosta *et al.* (2006) studied the expression of the VHSV G protein on the surface of RTG-P1 cells and its induction of type I IFN in neighbouring cells. Of the cells transfected with the pcDNA3 vector including the VHS G gene, 1–3% produced IFN due to the expression of the G protein on the cell surface, but cells transfected with plasmid alone were negative. When cells were transfected in the presence of MAbs to G protein, production of IFN was reduced by 50%. This indicated the surface expression of G protein was the main mechanism of IFN induction.

The kinetics of Mx expression in rainbow trout and Atlantic salmon were studied by Acosta *et al.* (2005). Duration of MxRNA expression to i.m. injection of VHSV G gene DNA vaccine was followed and compared with control plasmid in 24 g rainbow trout at 14°C over 11 weeks. Mx was detected on day 7 at first and peaked at day 14, then returned to pretreatment levels on day 21. In rainbow trout fingerling of 13 g and salmon parr of 12 g at 10°C, a rapid response to poly I:C was detected from day 1, which peaked from days 3–9 and decreased to background by day 12. The response to VHSV/DNA vaccine was slower to begin with; in salmon parr, the peak level was on day 6 and the signal disappeared by day 12, while in rainbow trout the peak was on day 12 and lasted until day 21. The authors concluded the duration of the Mx response was similar to the duration of the non-specific protection produced following vaccination with rhabdovirus DNA vaccines. This conclusion was corroborated by an earlier paper, which showed that Mx interfered with the replication of VHSV at the cell culture level (Caipang *et al.*, 2003).

The simultaneous expression of three isoforms of Mx 1, 2 and 3, in response to VHSV G gene, poly I:C or VHSV, was reported by Tafalla *et al.* (2007). This was the first report to show Mx induction after cell transfection with a plasmid coding for the VHSV G protein. Regardless of the inducer used, while in RTG-2 cells Mx3 was induced predominantly, in head kidney leucocytes all three isoforms were induced. *In vivo* in rainbow trout, a predominant expression of Mx3 transcripts was observed in muscle tissue, but expression of all three isoforms, predominantly Mx1 and 2, was found in head kidney and spleen. Thus, the expression of different Mx genes was shown to be regulated differentially *in vitro* and *in vivo*.

Cellular innate immunity

The literature on the innate cellular immune responses in fish is sparse, i.e. natural killer (NK) cells, non-specific cytotoxic cells (NCCs) and antibody-dependent cytotoxic cells. In gilthead sea bream (*Sparus aurata*), Esteban *et al.* (2008) showed for the first time that NCC and NK cell activity was enhanced in i.p. injected fish in a short time of under 48 h. NK-like, reactive oxidative intermediate (ROI) and myeloperoxidase (MPO) activity generally increased in head kidney (HK) and peritoneal exudate (PE) leucocytes with VHSV infection. These data showed that, in a reservoir species where the virus could not be detected in the spleen or liver at 72 h post-infection, virus could be detected by nested PCR in HKLs and PELs at 4 h and 72 h as strong evidence that leucocytes could endocytose the virus and clear it from the circulation.

Adaptive immunity

Lorenzen, N. *et al.* (1999) reviewed immunity to VHS in rainbow trout with particular emphasis on the adaptive immune response and vaccination.

Humoral/specific antibody

Neutralizing antibodies have been described for trout recovering from VHS infection. The assay is dependent on trout complement in normal serum for effective neutralization (Dorson and Torhy, 1979). An interesting facet to the assay, as pointed out by de Kinkelin (1988), is that neutralization is progressive and dependent on the time of incubation of antibody and virus. Incubation for 16 h at 4°C increases the serum neutralization end point value by 20 compared with that obtained for 1 h at 20°C.

Neutralizing antibody responses take a variable time to develop. Olesen *et al.* (1991) found that 130 g trout exposed to VHSV Voldberg strain by cohabitation developed antibodies 4–10 weeks later. Other assays for antibody, such as immunofluorescence (IF) and ELISA, detected antibody at different starting times and there was no strict correlation between positivity in the three assays.

Temperature plays a profound role in the development of the immune response, as reported by Vestergård-Jørgensen (1982) in the trout response to the Reva live vaccine strain. Basically, a low temperature of 5–10°C was optimal in stimulating a good priming of the immune response. In contrast, a too rapid clearance of the virus at high temperatures (15–20°C), involving IFN production, mitigated against the antibody response, giving a very low titre of antibody.

Fregeneda-Grandes *et al.* (2009) examined the antibody response to double viral infections with a similar virus, i.e. VHSV, and the genetically related infectious haematopoietic necrosis virus (IHNV). In a group of rainbow trout infected with both viruses, 30% of fish showed neutralizing antibodies against VHSV, 21% against IHNV, but 12% against both viruses. Thus, it was proved that fish could mount an antibody response to more than one virus at the same time.

Cell-mediated immunity

Lorenzen, N. *et al.* (1999) had reviewed this field briefly and commented, 'Little is known about cellular antiviral defence mechanisms in trout due to the lack of well-defined marker for leucocyte subpopulations, trout lymphocyte cell lines and syngenic trout.' As an indicator of an adaptive anamnestic response, Chilmonczyk (1978) showed that leucocytes from most VHS survivor trout proliferated on stimulation with inactivated virus, and later Lorenzo *et al.* (1995) showed stimulation with the G and N proteins, as well as synthetic peptides from these proteins. Clearly, this is an area for further work in a wide range of hosts to understand better the cell adaptive immune response to VHSV.

Control, Treatment and Epizootiology

Viral haemorrhagic septicaemia virus has a widespread distribution in a variety of wild and farmed freshwater and marine fish, and the host range as discussed above has also been reviewed by Skall *et al.* (2005). Aspects of control, treatment and factors associated with disease outbreaks follow.

Transmission control

Shedding and transmission of the virus take place with lateral spread of the disease from infected fish farms and development of the carrier state in nearby wild fish. This is evident in several European states where reinfection of farm stocks has taken place after eradication of the VHS infection on the farms. Shedding via the urine from clinically infected rainbow trout has been demonstrated as the most likely means of viral dissemination (Neukirch, 1985). However, the degree of viral shedding from persistently infected fish is more conjectural and has not been documented.

Official programmes of health control: EU zone-free status system

Fish farmers can attempt to avoid VHS infection by starting with approved stock free of the virus. However, the risk of VHSV

from wild stock remains for farms not on spring or borehole water. A risk-based surveillance programme is currently in place for EU member states in terms of avoiding VHS disease and stock assessment for the virus (European Commission Council Decision, 2006/88/EC).

Control at the farm level: disinfection of equipment and input water

A further possible future barrier to spread is the use of UV radiation to inactivate virus in the inflowing water. Dosage rates must be sufficiently high to inactivate VHSV at the rate of $1–3\times10^3\,\mu W/s/cm^2$ (Yoshimizu *et al.*, 1986), by analogy with virus-killing effects of UV for IHNV. Øye and Rimstad (2001) reported the UVC sensitivity in different units, a 3-log reduction being obtained by $7.9\pm1.5\,J/m^2$. Active methods of control are therefore represented by avoidance in practical terms.

Host factors in genetic control

An interesting genetic approach to VHS control was initiated by Dorson and Chevassus (1985). They showed that hybrids of either male coho salmon or brook trout×rainbow trout females could be made viable as triploid stock by heat shock after fertilization of the ova. Such triploid hybrids were far less susceptible to VHSV than either the diploid or triploid rainbow trout. This outcome shows that host susceptibility of rainbow trout as the most sensitive species to VHSV has a profound genetic basis, manifest in the degree of uptake of virus and the rate of virus multiplication in the target organs, namely the kidney and liver.

De Kinkelin (1988) put forward the view that exploitation of the brook trout×rainbow trout hybrid was of value in rainbow trout stocking in Continental Europe. Until a commercial vaccine is available for VHSV in Europe, a triploid hybrid breeding programme might offer great benefits to the trout industry.

Quillet *et al.* (2007b) reported a wide range of susceptibility, from 0–99%, of nine homozygous clones of rainbow trout to bath infection with VHSV strain 07-71. IHNV susceptibility was examined as well and, although the VHSV-resistant clones were not fully resistant to IHNV, the authors suggested that non-specific mechanisms common to both viruses were involved, because the susceptibility to both viruses was broadly correlated. The authors supported this strategy as warranted because it 'should improve the average innate immune reponse' of new fish strains against a background of the high mutation and evolution rate for RNA viruses. Lorenzen, N. *et al.* (1999) and Ellis (2001) also reviewed genetic immunity briefly and the interested reader should consult these papers.

Chemotherapy/antivirals

There is one recent report of an antiviral compound that acts against VHSV in cell culture. A synthetic human alpha-defensin-1 peptide of 30 peptides was reported to inactivate VHSV virions by two probable routes: firstly, through interfering with the G protein-dependent fusion with the cell membrane and, secondly, by inhibiting virus replication in target cells by upregulating genes related to type I interferon (Falco *et al.*, 2007).

Treatment and protection

Physical methods

Physical methods exist to inactivate VHSV in the water milieu and the equipment that contains the fish. An immediate entry portal for the virus, the input water, can be inactivated with UV light to prevent virus entry.

Disinfectants, such as chlorine, hypochlorite and iodophors, are very effective in killing rhabdoviruses and will prevent infection of transport tanks and equipment from farm to farm (Jørgensen, 1974). Prevention must be practised in control, as no therapy or chemotherapy exists for VHS disease.

Other means of protection – immune potentiators

The fact that tissue interferon (IFN) is developed in VHSV infected fish (de Kinkelin and Dorson, 1973) indicates that potentiators of IFN synthesis in fish may help to alleviate the disease and the extent of its spread. Immunostimulants could also have a beneficial effect on VHS disease. Peddie *et al*. (2003) showed that a trout-interleukin (IL)-1β-derived peptide, P3, consisting of 10 amino acids synthesized to complex with the IL-1 receptor, reduced the bath challenge mortality of an unstated strain of VHSV to 10 g rainbow trout.

Vaccination

Progress with vaccination

The history of VHS vaccination features a period of research into the testing of candidate vaccine strains, either killed or attenuated, from the mid-1970s through the 1980s up to 1988, when de Kinkelin (1988) reviewed the subject. Following the reluctance of any of the policy regulating authorities to accept a live attenuated strain for licensing in the field, there was an increased effort by several laboratories to produce an efficacious subunit or single virus-protein vaccine using recombinant DNA technology.

The essential requirements of a VHS vaccine were summarized by de Kinkelin (1988), as listed below:

1. Potent and efficacious for the life of the fish without a booster natural infection.
2. Safe to use and avirulent.
3. Cross-reacting against all strains, i.e. polyvalent in protection.
4. Cheap to buy.
5. Easy to use, ideally taken up in food.
6. Stable in storage and transport.
7. Providing effective resistance to the virulent disease, although persistent infection would be tolerated.
8. Not disseminated in wild fish or hindering epizootiological surveillance of control policies.

Killed vaccine

This type of vaccine was evaluated by de Kinkelin (1988). Strain 07-71 was inactivated by P-propriolactone and formalin at dilutions of 1/5000 and 1/2000, respectively. At 10°C, intraperitoneal injection and not water bathing was essential to promote an immune response; the range of protection in 5 g fish was from 30 to 100 days at 10°C or below. Cross-protection between three serotypes was shown and a degree of virus-neutralizing antibodies in the sera of vaccinated fish was detected.

Live vaccines

Vestergård-Jørgensen was the Danish pioneer of this approach (Jørgensen, 1976; Vestergård-Jørgensen, 1982). The Reva strain (related to the F1 reference strain) was atttenuated by 240 successive subcultures in RTG-2 cells at 14°C. It was shown to be a genetically stable strain after 20 backpassages in rainbow trout fry, when the 6-week mortality in 3 g rainbow trout fry changed by only 1% from 16 to 17%. Testing of this strain by immersion of fry in $\log_{10}$ 4.0 plaque-forming units (pfu)/ml water for 1 h at an optimum temperature below 10°C showed a protective effect up to 150 days after vaccination.

De Kinkelin and Béarzotti-Le Berre (1981) described the attenuation of an F1-related strain by subculture at a high temperature of 25°C in EPC cells, and this was termed the F25 strain. The attenuation by repeated cell-culture passage was induced by the cell and not temperature *per se*, which acted as a marker of the attenuation. An average increase in survival of immunized rainbow trout fry of 30% could be obtained, in comparison with non-immunized fry, when the challenge virulent virus dose was given. This protection was due largely to IFN, as the protection was also effective when the vaccine was given 24 h before challenge. However, further work showed that a neutralizing antibody response was made to the immunizing variant virus, but this was not protective against the wild-type virulent virus.

Subunit vaccines

The virus envelope protein, the G or glycoprotein, is believed to contain major virulence factors. Using recombinant DNA technology, the whole or parts of the G protein can be made and tested for vaccine potential (Lecocq-Xhonneux *et al.*, 1994). At present, it is not established to what degree the different arms of the adaptive immune response, humoral and cell-mediated, are necessary to achieve effective protection, or, indeed, if only the G glycoprotein is needed to achieve protection. There is no published information on either of these two areas and these are topics for future research on the fish immunological response to VHSV proteins.

Two major advances towards synthesizing subunit vaccines have been described. The Danish group at Aarhus cloned and sequenced the gene encoding the G protein of a recent Danish field isolate of VHSV (Lorenzen *et al.*, 1993). The major part of this protein, without the leader segment, was expressed in *Escherichia coli* as a protease–cleavage fusion protein. When this protein was renatured and purified, it could simulate VHSV-specific antibodies (assayed by neutralization) when injected into rainbow trout.

The next important subunit vaccine advance was made by the Eurogentec-led group (Lecocq-Xhonneux *et al.*, 1994). A recombinant subunit vaccine was made by expressing the glycoprotein in insect cells, using a baculovirus vector. This system was chosen because the insect cell and baculovirus system offered complete glycosylation of the G proteins, known to be important for the correct immunogenicity and three-dimensional folding of the G protein (Lorenzen *et al.*, 1993). The baculovirus-encoded protein was shown to induce the synthesis of virus-neutralizing antibodies in trout and to stimulate protection against challenge virus, but only when delivered by the intraperitoneal route. This was promising evidence that the glycoprotein could stimulate a specific immune response, but the failure to achieve protection after water bathing was a shortfall for practical vaccination of young trout. This represented one of the stated vaccination criteria of de Kinkelin (1988).

DNA vaccination against VHSV has been shown to be extremely effective for inducing a protective immune response under experimental conditions in both rainbow trout and Japanese flounder (Lorenzen *et al.*, 1998, 2001; Byon *et al.*, 2006). The standard protocol is to inject a small amount of plasmid DNA encoding the G protein (e.g. 1 μg to 0.5 g fish; Lorenzen *et al.*, 2001) intramuscularly. Very high relative protection survival was found, 98% and 85%, at 9 and 71 days postvaccination, respectively. As to the mechanism of action, it is reported that VHSV DNA vaccine induces not only the production of antibodies in the longer term (from 4 to 8 weeks postvaccination in rainbow trout; Ellis, 2001, quotes McLauchlan, unpublished) but also the expression of Mx genes in the muscle at the site of injection (Boudinot *et al.*, 1998). Despite the excellent protection of DNA vaccination, the technique of injection does not permit mass farm vaccination for lack of convenience.

An oral vaccine for immunization of rainbow trout against VHSV has been developed and tested experimentally which opens up the possibility of mass vaccination at farms (Adelmann *et al.*, 2008) because it overcomes the acid sensitivity of the virus in the fish stomach. By design, the vaccine was a polyethylene glycol (PEG)-coated bait, prepared by cold extrusion, measuring approximately 2×3 mm diameter, and the strain was the attenuated ATT 150 with an average titre of $1\times10^{5.5}\,TCID_{50}/g$. When delivered orally, this vaccine was taken up well by the gut, as assessed by an IFAT for VHSV antigen on gut sections. The vaccine led to higher expression levels of MHC class II and CD4 mRNAs when compared with control guts, measured using real-time PCR. Upregulation of these genes is known to be a first step in the activation of CD4+ T-helper cells (Th2) in the early phase of the immune response. Antibody responses were detected 5 weeks after vaccination in fish immunized i.p. and orally, but there was large individual fish variation, which might reflect

individual variation in the stimulation of antibody production. The relative protection survival produced was extremely high at 90% for the i.p. route and 80–84% for the oral route for a challenge strain producing around 60% mortality. The authors speculated that the protection of the vaccine was due to a potent stimulation of the cellular immune response rather than antibody stimulation, which can be proven by further investigation.

Factors associated with disease outbreaks

A discussion of key factors associated with VHS disease centres on estimating the risk of virus introductions, knowing the likelihood of different types of introductions and the consequences of disease. The recent history of outbreaks of VHS in the Great Lakes in North America for genotype IVb (Elsayed *et al.*, 2006), Norway for genotype III (Dale *et al.*, 2009) and Finland for genotype 1 (Raja-Halli *et al.*, 2006) suggests that VHSV has a great capacity to adapt to new hosts. The recent apparent import of VHSV into England (Stone *et al.*, 2008) highlights the continued risk of exposing susceptible species to known virulent strains.

The key risks which may increase the opportunity for viral adaptation in new species or increased exposure of known susceptible species to virus enzootic in the wild population include: (i) mixed species farming; (ii) wet feed diet; (iii) exotic introductions; and (iv) pump ashore and sea pen sites in relation to marine carriers. In relation to mixed species, farming of rainbow trout in fjords or seawater lochs near to farmed cod or halibut or other carrier species could risk viral transfer to farmed rainbow trout. With respect to a wet feed diet risk for marine species, minced Atlantic herring or Norway pout could have a definite risk for viral introduction. Raja-Halli *et al.* (2006) attributed early isolation of genotype I isolates in Finland to trash fish wet feed diets. Considering exotic introductions, trade movement of eviscerated fish could have finite risk of viral transfer via the muscle and flesh of VHS carriers, but more research is needed in this area. In relation to pump ashore/sea pen sites and 'situational risk', the outbreak of VHS in turbot in Gigha Island, Scotland, in 1994 (Ross *et al.*, 1994) was caused by a genotype III isolate. Subsequently, naturally occurring genotype III isolates, which were shown experimentally to cause disease in turbot, were identified in the marine environment of the British Isles (Snow *et al.*, 2005). In this case, exposure of a susceptible species to an enzootic virus, rather than specific adaptation, highlights a risk of farming known VHS-susceptible species in areas where the virus is present. The genotype III isolate from Norwegian rainbow trout (Dale *et al.*, 2009) in the west of Norway in the Storfjorden was attributed to an unknown marine source, but might represent the first adaptation of genotype III marine virus to this species.

Recommendations for Future Studies

1. Vaccine delivery vehicles for a universal vaccine that is efficacious for global genotypes I–IV.
2. Host receptors for VHSV in susceptible and carrier host fish species.
3. Viral sequestering and persistence in carrier and reservoir fish species.
4. Cellular adaptive and innate immunity and its importance in susceptibility and resistance.
5. Viral shedding of different genotypes in aquarium pathogenicity experiments and quantification of the minimum infectious dose by bath experiments.
6. The role of immunostimulants and neutraceuticals in mitigating viral infection and clinical VHS.

Acknowledgements

We thank Dr David Bruno for the photographs of Figs 3.1, 3.2, 3.3, 3.5, 3.6 and 3.7 and Jimmy Gauld for the photograph of Fig. 3.4.

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4 Infectious Salmon Anaemia

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Introduction

Infectious salmon anaemia (ISA) is a viral disease that was first recorded in Norway in 1984 in Atlantic salmon (*Salmo salar*) (Thorud and Djupvik, 1988). The disease has only been detected in Atlantic salmon under natural conditions. Diseased fish in the terminal stage are severely anaemic, hence the name of the disease. Initially, the disease spreads in a pattern consistent with a contagious disease, which has also been confirmed experimentally (Thorud and Djupvik, 1988; Thorud, 1991; Dannevig *et al.*, 1994; Fig. 4.1). The ISA epidemic in Norway peaked around 1990 when ISA was detected in more than 80 fish farms. Together with other significant disease problems present at that time, like furunculosis, this prompted the national fish health authorities to implement various biosecurity actions during the period 1988–1991. These included mandatory health control in hatcheries, mandatory health certification, ban on use of seawater in hatcheries, ban on movement of fish already stocked in seawater, regulations on transportation of live fish, regulations on disinfection of wastewater from fish slaughterhouses and of intake water to hatcheries. Later, the segregation of generations of fish was made mandatory, but had been a common practice from the same period (ISA contingency plan). The result of these actions was a general improvement of the sanitary situation in the fish farming industry and, together with significant improvements in husbandry practices, better laboratory identification and subsequent restrictions put on farms with ISA, a remarkable and rapid reduction in the number of ISA outbreaks was obtained (Håstein *et al.*, 1999). In latter years, the number of annual ISA outbreaks has been between 3 and 20 in Norway, while salmon production has increased at least sevenfold during the period after the ISA epidemic peaked.

ISA was reported from 1997 to 2000 in farmed Atlantic salmon in Canada (New Brunswick and Nova Scotia) and the USA (Maine), in Scotland and in the Faeroes (Rodger and Richards, 1998; Lovely *et al.*, 1999) . In Scotland, Canada, the USA and the Faeroes, the disease has not been present since 2008; however, detection of ISAV from the gills of salmon showing no signs of ISA is common (Faeroes) and has been reported from Scotland (Anon., 2005).

In 2007, several typical ISA outbreaks were reported in Chile in Atlantic salmon in seawater, and a large fraction of the production sites has been affected by the epidemic (Godoy *et al.*, 2008). As of 2008, the ISA situation in Chile has many similarities to that of Norway in the early 1990s.

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Viral, Bacterial and Fungal Infections, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

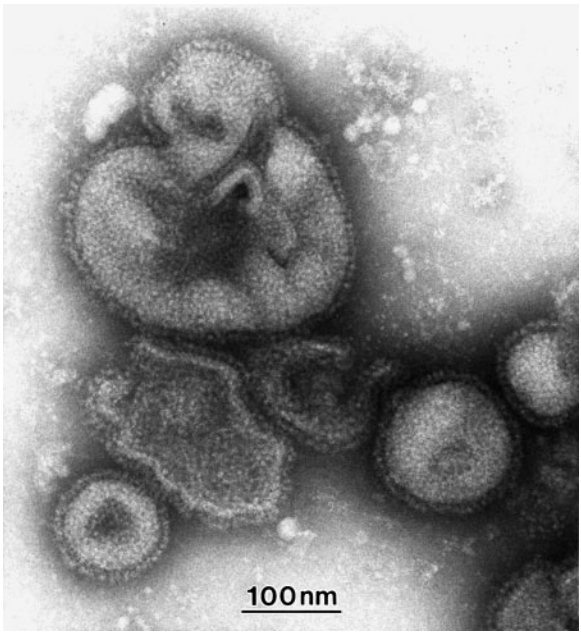


Fig. 4.1. Electron micrograph of negative-stained ISAV particles purified from infected cell culture medium. Photograph by Ellen Namork, Norwegian Institute of Public Health, Oslo, Norway.

Disease Characteristics

A large majority of natural ISA outbreaks in farmed Atlantic salmon have occurred in the seawater stage. Very few outbreaks have been reported in the freshwater phase, but nevertheless ISA has been reported in yolk-sac fry (Nylund *et al*., 1999). In Atlantic salmon kept in fresh water, experimental ISAV infection is induced readily and transmits easily between individual fish (Dannevig *et al*., 1994; Rimstad *et al*., 1999).

Before the introduction of mandatory depopulation of net pens and production sites experiencing ISA outbreaks in Norway, the cumulative mortality during an outbreak could exceed 90% after several months. However, daily mortality seldom exceeds 0.05–0.1% in affected net pens.

In the initial stages of an outbreak, a slightly increased mortality is often seen and the disease outbreak frequently develops very slowly over several weeks. In many cases, increased mortality can be related to stress situations such as handling of the fish and delousing. The disease usually starts in one net pen and it may take weeks and even months before clinical disease develops in neighbouring net pens, indicating that ISA is not a fast-spreading disease. In a disease outbreak with low mortality, the clinical and macroscopical changes may be limited to anaemia and some circulatory disturbances. Immunohistochemistry demonstrates a systemic infection confined mainly to endothelial cells. The chronic disease phase with low mortality can be overlooked easily if no diagnostic investigations are undertaken. Occasionally, episodes of acute high mortality over a couple of weeks may ensue, especially if no measures are taken. The pathology is then more severe, with ascites and haemorrhage dominating. The categorization of disease outbreaks into acute and chronic is a simplification, as transitional forms are often present. The disease appears throughout the year, but outbreaks are detected more frequently in spring/early summer and in late autumn.

Seasonal variations in mortality have been experienced in controlled challenge experiments with ISAV on Atlantic salmon. Generally, a chronic progression of the disease occurs during the autumn, often with low cumulative mortality. However, during

the spring, an acute progression has normally been observed. This could indicate that there are seasonal and host-related factors that are of importance to the outcome of the infection. It has been found that the susceptibility of Atlantic salmon towards ISAV infection increases when the fish is in the process of smoltification (Glover *et al.*, 2006). Thus, it could be the physiological status of the fish, and not the season of the year, that is linked to disease progression. There were suggestions that farmed Atlantic salmon were more susceptible than wild Atlantic salmon, but no differences between farmed, wild and hybrid fish to ISAV infection have been found (Nylund *et al.*, 1995; Glover *et al.*, 2006).

In natural outbreaks, the incubation time in a cage or farm may be variable. It is therefore difficult to pinpoint the time of the introduction of the virus to the farm. The incubation time in natural outbreaks is estimated to be from a few weeks to several months (Vagsholm *et al.*, 1994; Jarp and Karlsen, 1997). In experimental challenges by injection, the disease appears normally after 2–3 weeks (Thorud and Djupvik, 1988; Thorud, 1991; Dannevig *et al.*, 1994; Rimstad *et al.*, 1999). In non-injected cohabitants, there is normally approximately a one-and-a-half-week delay before disease develops. Daily mortality in a farm generally stays low, but often increases to a more significant level (0.05–0.1%). Without intervention, the cumulative mortality may become very high.

Clinical features

Diseased fish are usually in normal nutritional condition but swim sluggishly in the water surface or hang listlessly at the net-pen wall. A progressive anaemia that may result in a 'watery blood' (haematocrit < 10) normally occurs. Prominent clinical findings may include pale gills, localized haemorrhage of eyes and skin, exophthalmia and scale oedema, suggesting circulatory disturbances (Thorud and Djupvik, 1988; Evensen *et al.*, 1991; Thorud, 1991; Koren and Nylund, 1997; Essbauer and Ahne, 2001). It should be noted that the disease may appear in different manifestations and development may progress from an acute to a slowly developing chronic disease. As almost all infectious diseases, infectious dose, virus strain, environmental and management factors and genetic make-up of the host will influence disease development.

Pathology and pathogenesis

ISA is a systemic disease affecting the blood and the circulatory system. The major target cells for the infection are endothelial cells lining blood vessels of all organs, including sinusoids, endocardium and endothelial macrophages (Hovland *et al.*, 1994; Koren and Nylund, 1997; Falk *et al.*, 1998; Gregory, 2002; K. Falk and O.B. Dale, personal observation, 2008). The final stages of the disease are characterized by pathological changes consistent with a circulatory collapse and include an extreme anaemia (haematocrit < 10), ascites, oedema and bleeding and necrosis in one or several organs. Though virus-infected endothelial cells are observed in all organs using immunohistochemical techniques, a striking observation is the limited inflammatory cellular response.

On autopsy, the pattern of haemorrhage may be present in the liver, kidney, gut and gills. The spleen is more constantly swollen and dark (Mjaaland *et al.*, 1997; Devold *et al.*, 2000; Mikalsen *et al.*, 2001; Snow *et al.*, 2003a; Anon., 2006), the liver is dark in colour due to haemorrhagic necrosis (Evensen *et al.*, 1991) and the lesions result in extensive congestion of the liver with dilated sinusoids and, in later stages, the appearance of blood-filled spaces. By electron microscopy of experimentally infected fish, changes involving the perisinusoidal macrophages were observed from 4 days post-infection (d.p.i.) (Speilberg *et al.*, 1995), degenerative features of the sinusoidal endothelium at 14 d.p.i. and, by 18 d.p.i., areas of the liver

were devoid of a sinusoidal endothelial lining. Gross and light microscopic changes were first recorded at 18 d.p.i., as was a significant decrease in the haematocrit values. By 25 d.p.i., characteristic multifocal, confluent, haemorrhagic necroses were present (Speilberg *et al*., 1995). The ISA-typical gross macroscopic liver changes are thus present late in the disease development. Immunohistochemistry of the liver shows infection of the endothelial cells only, and this appears to precede the haemorrhagic necrosis (O.B. Dale, personal observation, 2008).

The kidney manifestation is characterized by moderately swollen tissue with interstitial haemorrhaging and some tubular necrosis (Byrne *et al*., 1998; Simko *et al*., 2000). The gut is characterized by dark red walls due to haemorrhaging within the intestinal wall, but not to the gut lumen (in fresh specimens). The gill manifestation is an exception to the pale anaemic gills, as blood accumulates especially in the central venous sinus of the gill filaments. The haemorrhagic organ lesions are easily visible on autopsy in organs like liver and gut, and less obvious in gills and kidney. In an ISA outbreak, one of the haemorrhagic organ manifestations can dominate, while in other outbreaks all manifestations can be found and, even in a single fish, all manifestations may, to some extent, be found. Outbreaks dominated by either the liver or kidney manifestation appear most common. However, the haemorrhagic organ lesions can be absent or very rare in the initial stages of an ISA outbreak, leaving only anaemia and the more subtle circulatory disturbances as a clue to the aetiology (Fig. 4.2).

In the more slowly developing chronic forms of ISA, clinical signs and pathological changes may be more subtle. The liver may

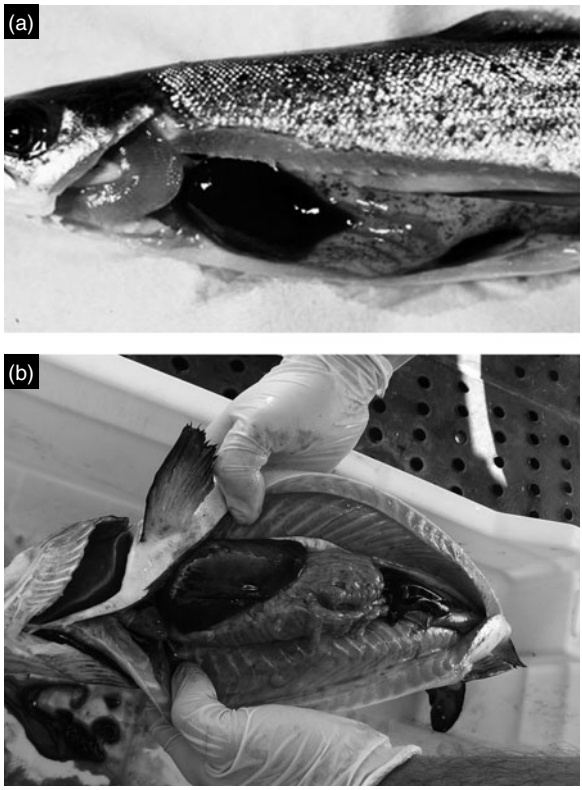


Fig. 4.2. Atlantic salmon with findings typical of ISA. (a) Pale gills and muscle, petechial haemorrhage, dark liver and spleen, ascites are all present. (b) Dark liver, dark and swollen spleen are prominent, petechial haemorrhage and paleness are not prominent. Photographs: Trygve Poppe, Norwegian School of Veterinary Science, Oslo, Norway.

appear pale or yellowish and the anaemia may not be as severe as in the acute disease. There is reduced ascitic fluid, but haemorrhage in the skin and swim bladder and oedema in the scale pockets and swim bladder can be more pronounced than in acutely diseased fish (Evensen *et al.*, 1991).

Studies of the mechanisms of ISAV infection indicate that the major port of ISAV entry is the gills, but other ports of entry cannot be excluded (Mikalsen *et al.*, 2001). In infection experiments, virus have been detected throughout the body 5–10 d.p.i., with a peak in viral load at approximately 15 d.p.i. (Rimstad *et al.*, 1999). This was followed by a temporary decrease in viral load and, after 25 d.p.i., a second rise in viral replication followed which continued to the terminal stage of ISA (Rimstad *et al.*, 1999; Mikalsen *et al.*, 2001).

Host Range

Atlantic salmon is the only species in which ISA occurs naturally. The virus can replicate in several other species, but no disease has been observed under aquaculture conditions (Table 4.1). However, these species may be important as carriers of virus and as reservoirs (Nylund and Jakobsen, 1995; Nylund *et al.*, 1995; Snow *et al.*, 2001b).

Wild Atlantic salmon are susceptible to ISA experimentally and show the same clinical signs as farmed fish (Raynard *et al.*, 2001a; Glover *et al.*, 2006), and ISAV is present in feral Atlantic salmon and sea trout (*Salmo trutta*) (Nylund and Jakobsen, 1995).

Replication of ISAV occurs in experimentally infected Atlantic salmon, brown trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), chum salmon (*O. keta*), coho salmon (*O. kitsutch*), herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) (Nylund and Jakobsen, 1995; Nylund *et al.*, 1997, 2002; Snow *et al.*, 2001a; Rolland and Winton, 2003; Grove *et al.*, 2007). ISA has been reported once in coho salmon in Chile (Kibenge *et al.*, 2001a), but it has not been reported elsewhere. None of the above-mentioned species develop clinical or pathological signs of ISA, with the exception of experiments in rainbow trout, where disease/mortality and/or pathological changes have been described (Biacchesi *et al.*, 2007; MacWilliams *et al.*, 2007). The latter could be ascribed to either high infectious doses of virus in experimental infections or to particular susceptible family groups of the fish used.

ISAV replicates in and has been found in feral trout, and this species could be a reservoir of the virus (Nylund *et al.*, 2003), though some of the reported findings could possibly be ascribed to ongoing ISA outbreaks in farmed salmon in the area (Plarre *et al.*, 2005). In Norway, sea trout are abundant in fjords and coastal areas, i.e. near fish farms, while the feeding areas of wild Atlantic salmon are some distance from the coast. The migratory behaviour of sea trout may explain the appearance of disease in fish farms located far from ISA-affected farms. However, it is not known whether the virus originates in sea trout or sea trout become infected by ISAV infected farmed salmon. It should also be mentioned that ISAV has been detected in farmed rainbow trout in Ireland, though no signs of disease were observed (Siggins, 2002).

In a challenge trial, various Canadian and Norwegian ISAV strains were injected intraperitoneally into Pacific salmon (*O. mykiss*, *O. keta*; *O. kitsutch*, *O. tshawytscha*) and the virus could be re-isolated, suggesting replication of the virus, but no mortality or signs of disease were noted. The conclusion was that Pacific salmon were resistant to ISA compared to Atlantic salmon, but that these species should not be ignored as potential virus carriers (Rolland and Winton, 2003).

In a comprehensive survey of marine fishes, alewife (*Alosa pseudoharengus*), American eel (*Anguilla rostrata*), Atlantic herring (*C. harengus harengus*), Atlantic mackerel (*Scomber scombrus*), Atlantic cod (*G. morhua*), haddock (*Melanogrammus aeglefinus*), Atlantic halibut (*Hippoglossus hippoglossus*), pollock (*Pollachius virens*), American shad (*Alosa sapidissima*) and

Table 4.1. Detection of ISAV in wild fish and replication of ISAV in different fish species after experimental infection.

Species	Systematic name	Wild fish	Experimental infection	Days after challenge	Detection method	References
Salmon	*Salmo salar*	Pos	Pos	>200	Several	Nylund *et al.* (1995); Raynard *et al.* (2001b); Plarre *et al.* (2005)
Brown trout	*S. trutta*	Pos	Pos	>200	Several	Nylund *et al.* (1995); Raynard *et al.* (2001a); Plarre *et al.* (2005)
Arctic char	*Salvelinus alpinus*		Pos	>40	RT	Snow *et al.* (2001a)
Rainbow trout	*Oncorhynchus mykiss*		Pos	>200	Several	Several authors
Chum	*O. keta*		Pos	>15	Cc	Rolland and Winton (2003)
Chinook	*O. tshawytscha*		Neg	15	Cc	Rolland and Winton (2003)
Coho salmon	*O. kitsutch*	Pos[a]	Pos	>15	RT, Cc	Kibenge *et al.* (2001a); Rolland and Winton (2003)
Halibut	*Hippoglossus hippoglossus*	Neg	Neg		RT	MacLean and Bouchard (2003)
Turbot	*Scophthalmus maximus*		Neg		RT, Cs	Hjeltnes (1993)
Herring	*Clupenga harengus*	Neg	Pos	>42	RT, Cs	Nylund *et al.* (2002)
Eel	*Anguilla anguilla*	Pos	Neg	7	RT	Stagg *et al.* (2001)
American eel	*Anguilla rostrata*	Neg				MacLean and Bouchard (2003)
Goldsinny	*Ctenolabrus rupestris*		Neg	7	RT, Cs	Hjeltnes (1993)
Cod	*Gadus morhua*	Neg	Neg/pos[b]		RT, ReTi	Grove *et al.* (2007); MacLean and Bouchard (2003); Snow and Raynard (2005)
Haddock	*Melanogrammus aeglefinus*	Neg			RT	MacLean and Bouchard (2003)
Pollock	*Pollachius virens*	Neg			RT	McClure *et al.* (2004)
Winter flounder	*Pseudopleuronectes americanus*	Neg			RT	MacLean and Bouchard (2003)
Mackerel	*Scomber scombrus*	Neg			RT	MacLean and Bouchard (2003)
Alewife	*Alosa pseudoharengus*	Pos			RT	MacLean and Bouchard (2003)
Saithe	*Pollachius virens*		Neg	<7	RT	Snow *et al.* (2002)
Sea bass	*Dicentraxus labrax*		Neg		Cs	Hjeltnes (1993)
American shad	*Alosa sapidissima*					
Blue mussel	*Mytilus edulis*	Neg[c]			RT, Cc	Skar and Mortensen (2007)
Salmon louse	*Lepeophtheirus salmonis*		Neg (pos)	<24 h	Cs	Rolland and Nylund (1998)

Cs, ISAV detected by challenging salmon; Cc, isolation of ISAV in cell culture; RT, ISAV detected by RT-PCR; ReTi, ISAV detected by real-time RT-PCR. [a]Farmed fish; [b]after injection of virus; [c]collected from a salmon farm positive for ISA.

winter flounder (*Pseudopleuronectus americanus*), no ISAV was found using RT-PCR. Cod and pollock sampled near ISA diseased salmon tested positive for ISAV (MacLean and Bouchard, 2003). Pollock cohabitating with farmed Atlantic salmon in sea cages remained RT-PCR negative when harvested together with salmon experiencing increased mortality due to ISA (McClure *et al.*, 2004). The same species was negative for ISAV following exposure by intraperitoneal injection of virus or by cohabitation with ISAV infected Atlantic salmon (Snow *et al.*, 2002). However, the fact that surveys have not yet revealed a non-salmonid reservoir does not prove that a reservoir does not exist.

There are no indications that ISAV can infect blue mussel (*Mytilus edulis*) and scallops (*Pecten maximus*) or that these shellfish play any role as a reservoir for ISAV (Skar and Mortensen, 2007).

Table 4.1 summarizes detection of ISAV in wild fish and replication of ISAV in different species. Based on the detection of ISAV by RT-PCR methods in wild fish, both *S. salar* and *S. trutta* are the most likely candidates as the natural hosts.

Characterization of ISAV

ISAV is classified as the type species of the genus *Isavirus* in the *Orthomyxoviridae* family (Fauquet *et al.*, 2005). The Atlantic salmon-derived cell lines, SHK, ASK, TO and As, but also the Pacific salmon-derived cell line CHSE-214, support the propagation of ISAV (Dannevig *et al.*, 1995b; Devold *et al.*, 2000; Kibenge *et al.*, 2000; Wergeland and Jakobsen, 2001; Rolland *et al.*, 2005). However, the CHSE-214 cell line does not support or only poorly supports growth of the European variants of ISAV and several laboratories have not been able to replicate ISAV in CHSE-214 cells at all, which may be ascribed to differences in the lines of CHSE-214 cells. The ASK cells are currently the preferred cell line for primary isolation and all known ISAV isolates replicate in these cells, with the exception of the HPR0 type, which has never been isolated in any cell line.

ISAV is a pleomorphic enveloped virus, 100–130 nm in diameter, with 10–12 nm surface projections (Dannevig *et al.*, 1995b; Eliassen *et al.*, 2000; Falk *et al.*, 2004). ISAV haemagglutinates erythrocytes from several fish species, though a notable exception is brown trout erythrocytes (Falk *et al.*, 1997). In addition, the virus also agglutinates erythrocytes from horses and rabbits. The buoyant density of virus particles in sucrose and caesium chloride is 1.18 g/ml. The virus is stable between pH 5.7 and 9.0, and it has a 3-$\log_{10}$ reduction in titre after 4 months when maintained in sterile seawater at 4°C (Rimstad and Mjaaland, 2002). The optimum temperature for replication in susceptible fish cell lines is 10–15°C. There is no replication in SHK-1 cells at 25°C or higher, and even at 20°C the yield of virus in SHK-1 cells is only 1% of the yield at 15°C (Falk *et al.*, 1997).

The genome consists of eight single-stranded RNA segments of negative polarity ranging in length from 1.0 to 2.3 kb and with a total size of approximately 14.3 kb (Mjaaland *et al.*, 1997; Clouthier *et al.*, 2002; Figs 4.1 and 4.3). The amino acid identity between the ISAV proteins and those of other orthomyxoviruses is low (13–25%) (Mjaaland *et al.*, 1997; Krossoy *et al.*, 1999; Kibenge *et al.*, 2001b; Snow and Cunningham, 2001; Clouthier *et al.*, 2002; Ritchie *et al.*, 2002). Reassortment of gene segments is a frequent occurrence in influenza A virus infection in birds and is, together with genetic drift, a major contributor to the evolution of these viruses and the emergence of new virulent strains. Molecular and phylogenetic sequence analyses of Norwegian ISAV isolates provide strong evidence that genetic reassortment also occurs for ISAV (Markussen *et al.*, 2008). Recombination events between segment 5 (fusion protein) and segment 3 (the nucleoprotein) have been documented (Devold *et al.*, 2006). The genomic segments are numbered according to size. The viral genome encodes at least ten proteins, where nine are known to be present in the mature virus particle. The two smaller segments (i.e. 7 and 8) each encode more than one protein. The *Orthomyxoviridae* is the only family of negative-stranded RNA viruses that are known to be

able to splice transcripts, which is made possible because the RNA replication occurs in the nucleus and, accordingly, one (or maybe two) group of mRNA transcribed from ISAV segment 7 is spliced.

The nucleotide sequences and deduced encoded proteins of all eight genome segments have been described in Table 4.2.

Major structural proteins

Four major structural proteins are found when viral proteins from purified viral particles are separated on gels (Falk *et al.*, 1997, 2004; Snow and Cunningham, 2001). These proteins have been found to be the haemagglutinin-esterase glycoprotein (HE), the fusion (F) glycoprotein, the nucleoprotein (NP) and the matrix (M) protein (Falk *et al.*, 2004).

Envelope glycoproteins

There are at least two virus-encoded glycoproteins embedded in the viral envelope. They form spikes projecting on the surface of the virus. In general, viral envelope glycoproteins are important immunogens. Moreover, the surface glycoproteins of orthomyxoviruses are necessary for entering and leaving the host cells, and thus are essential for cell and tissue tropism, and consequently are also for virulence and pathogenesis. Experimental infections using different ISAV isolates have suggested variation in virulence between isolates, and the surface glycoproteins are considered to be of particular importance in this respect (Mjaaland *et al.*, 2005; Kibenge *et al.*, 2006).

Three vital biological activities are related to surface proteins of influenza viruses. These are haemagglutinating activity mediating receptor binding, fusion activity mediating fusion between the viral and endosomal membranes, and thus entry of the ribonucleoprotein into the cytoplasm, and receptor-destroying activity (RDE). ISAV has both haemagglutinating activity (Falk *et al.*, 1997) and fusion activity (Eliassen *et al.*, 2000; Aspehaug *et al.*, 2005), as well

Table 4.2. Genomic segments and encoded proteins of ISAV.

Segment [kb]	Encoded protein	Protein [kDa]
1 [2.3]	Polymerase, PB2	80.5[a]
2 [2.3]	Polymerase, PB1	79.5[a]
3 [2.2]	Nucleoprotein, NP	66–74[b]
4 [2.0]	Polymerase, PA	65.3
5 [1.7]	Fusion, F	50–53
6 [1.5]	Haemagglutinin-esterase, HE	38–46[b]
7 [1.3]	Three open reading frames:	
	ORF1=non-structural (NS)	34
	ORF2=spliced mRNA	17
	ORF3=spliced mRNA[c]	11
8 [1.0]	Two open reading frames	
	Matrix, M	22–24[b]
	RNA-binding protein	26

[a]Estimations based on amino acid sequence. [b]The estimated molecular masses of some of the proteins differ slightly in the literature, probably due to differences in experimental conditions. For HE, this could also be due to differences in glycosylation, as well as in the highly polymorphic region (HPR); see text. [c]The existence of this mRNA has not been verified by all authors.

References: Segment 1 (PB2): Snow *et al.* (2003b); segment 2 (PB1): Krossoy *et al.* (1999); segment 3 (NP): Snow and Cunningham (2001); Aspehaug *et al.* (2004); Falk *et al.* (2004); segment 4 (PA): Clouthier *et al.* (2002); segment 5 (F): Aspehaug *et al.* (2005); segment 6 (HE): Krossoy *et al.* (2001); Rimstad *et al.* (2001); segment 7 (NS): Biering *et al.* (2002); McBeath *et al.* (2006); Kibenge *et al.* (2007); Garcia-Rosado *et al.* (2008); segment 8 (M): Mjaaland *et al.* (1997); Falk *et al.* (2004); Garcia-Rosado *et al.* (2008).

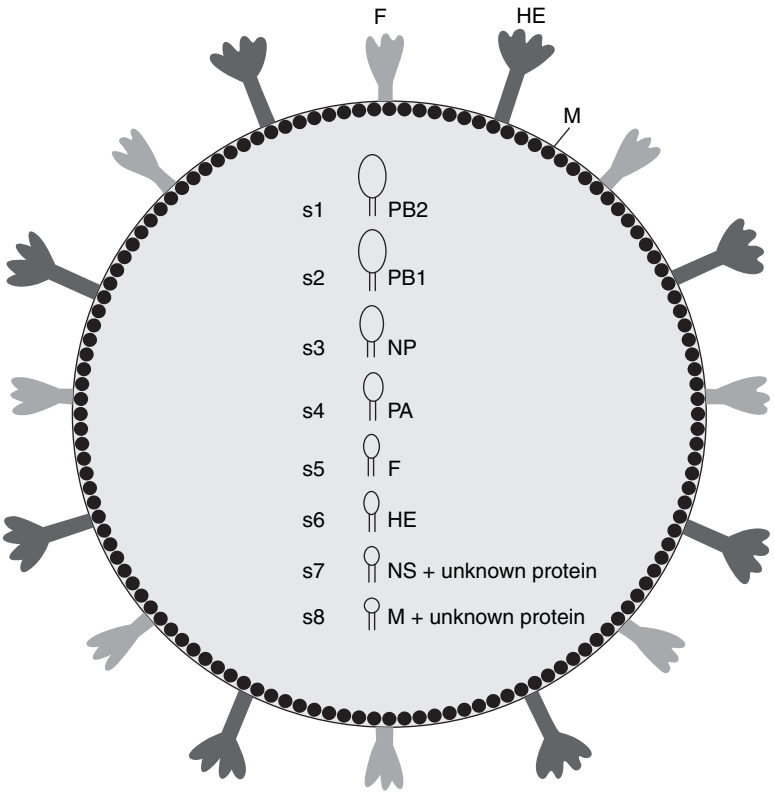


Fig. 4.3. ISAV particle showing segments 7 and 8 each encode two proteins, one protein from each of these two segments is yet to be named/characterized. PB2/PB1/PA, polymerase subunits; NP, nucleoprotein; F, fusion protein; HE, haemagglutinin-esterase; NS, non-structural protein; M, matrix protein.

as receptor-destroying activity, the latter being an esterase (Falk *et al.*, 1997, 2004). In contrast to the other orthomyxoviruses, but similar to most paramyxoviruses, the haemagglutinating and esterase activities are performed by the very same viral protein – the 38–46 kDa haemagglutinin-esterase (HE)-protein (Falk *et al.*, 2004) – while fusion activity is located on the other 50 kDa surface F-protein (Falk *et al.*, 2004; Aspehaug *et al.*, 2005).

Haemagglutinin-esterase glycoprotein

The haemagglutinin-esterase protein has been estimated to be 38–46 kDa in size and it is encoded by genomic segment 6. The observed size variation is assumed to be due to variation in the numbers of putative *N*-glycosylation sites, which may vary (from one to three) between isolates. Also, variation in the length of the highly polymorphic region (HPR) may influence the estimated size of HE. The main functions of the HE are receptor binding and the receptor-destroying enzyme (RDE), esterase, activity. It has been demonstrated that ISAV specifically binds to the acetyl group of 4-*O*-acetylated sialic acid, which is commonly found attached to the sugar chains of glycoproteins and glycolipids (Hellebo *et al.*, 2004). Correspondingly, the RDE specificity has also been shown to be 4-*O*-acetylated sialic acids; thus, specificity of receptor binding and RDE is different from that found with other known orthomyxoviruses. Receptor binding

and RDE can be demonstrated by haemagglutination assay. Erythrocytes from several fish species can be haemagglutinated by ISAV, as can erythrocytes from mammalian species like horse and rabbit (Falk *et al.*, 1997). Interestingly, ISAV do not haemagglutinate erythrocytes from brown trout (*S. trutta*). RDE activity can be demonstrated by elution of ISAV agglutinated erythrocytes, following prolonged incubation of the reaction, and is found with most species' erythrocytes, with the exception of Atlantic salmon. The protein probably exists as a tetramer in the viral membrane.

One significant feature of the ISA HE not found in other orthomyxoviruses is the highly polymorphic region (HPR) just upstream of the transmembrane domain of the protein, i.e. at the stem region of the HE spike (Devold *et al.*, 2001; Krossoy *et al.*, 2001; Rimstad *et al.*, 2001; Mjaaland *et al.*, 2005). Variation in this region is characterized by the presence of gaps rather than single-nucleotide substitutions and more than 25 aa patterns or HPR types ranging in length from 11 aa to 35 aa have been described (Devold *et al.*, 2001; Kibenge *et al.*, 2001b; Mjaaland *et al.*, 2002). The ORF of the *HE* gene varies in length from 1188 bp to 1152 bp nucleotides, depending on the length of the HPR (Devold *et al.*, 2001). The polymorphism has been suggested to arise from differential deletions of a full-length precursor gene (HPR0) as a consequence of strong functional selection. All HPRs described to date can be explained to have derived from such a full-length sequence (Cunningham *et al.*, 2002; Mjaaland *et al.*, 2002; Nylund *et al.*, 2003). Virus with HPR0 has never been isolated in culture, but has been detected in tissue, mainly in gills, by RT-PCR and has never been associated with classical ISA (Cunningham *et al.*, 2002; Cook-Versloot *et al.*, 2004; Nylund *et al.*, 2007). Thus, the HPR0 type has been suggested to be non- or low virulent. It has also been suggested that virus with short HPR are more virulent (Kibenge *et al.*, 2006); however, whether there is a connection between the size or type of the gap and virulence and how deletions in the HPR influence viral virulence remain to be determined.

Atlantic salmon that have survived an experimental ISA infection are less susceptible to reinfection, and convalescent sera partly protect against ISA in infection experiments (Falk and Dannevig, 1995), indicating the production of antibodies against ISAV. Furthermore, the same study shows that neutralizing antibodies may be produced. Antibodies have not been found to inhibit haemagglutination and only a minor inhibition of the haemadsorption was found when HE expressing cells were pretreated with convalescent salmon serum (Mikalsen *et al.*, 2005). Although Atlantic salmon produce neutralizing antibodies against ISAV, the neutralization titres are generally low (Mikalsen *et al.*, 2005). A major part of the humoral response was directed against the NP (Western immunoblot) and, to a lesser extent, against the HE (K. Falk, personal observation, 2008), while reconvalescent sera only recognized NP-expressing cells to a minor extent (Mikalsen *et al.*, 2005). The low neutralization and haemadsorption-inhibiting activity of the salmon sera imply that interference with the receptor binding site is not as important a target for the salmon antibodies as the corresponding humoral response is for mammals.

Fusion glycoprotein

The fusion (F) protein is a 50 kDa glycoprotein encoded by genomic segment 5 and is activated by proteolytic cleavage at R267 or K276 (Falk *et al.*, 2004; Aspehaug *et al.*, 2005; Markussen *et al.*, 2008). It is synthesized as a precursor, F0, which may be cleaved into the disulfide-linked fragments F1 and F2. It is thus a type I fusion protein (Aspehaug *et al.*, 2005). Just downstream of the cleavage site, there is a hydrophobic sequence called the fusion peptide. The cleaved F protein is in a metastable state and fusion activity can be triggered by low pH (Aspehaug *et al.*, 2005). The active state of the protein probably exists as a trimer. Presence of the ISAV HE improves fusion

efficacy significantly and might be required in the fusion process.

Many ISAV isolates have been shown to possess inserts of 8–11 amino acids just upstream of the putative cleavage sites (Devold *et al.*, 2006; Markussen *et al.*, 2008). Three different inserts have been suggested to originate either from other parts of segment 5 or from the genomic segment 3. The significance of these inserts for function and virulence has not yet been determined. It can be speculated whether these inserts, together with the deletions in the HE HPR region, may influence the physical interaction between these two proteins and thus be of significance for virulence. In addition to this, it has also been suggested that a Q266 to L266 substitution is a necessity for virulence, unless there is a sequence insertion near the cleavage site (Markussen *et al.*, 2008).

Nucleoprotein

The NP is a 66–74 kDa phosphoprotein encoded by genomic segment 3 (Aspehaug et al., 2004; Falk *et al.*, 2004). The protein has been found to bind RNA (Aspehaug *et al.*, 2004). It is assumed that ISAV NP, like the other orthomyxoviral NPs, interacts with the viral RNA together with the three subunits of the RNA-dependent RNA polymerase (PB1, PB2 and PA) to form the inner core, known as the viral ribonucleoprotein (RNP) complex, and that NP directs this complex to the nucleus on arrival in the cell. Helical RNP-like particles, similar to those described for influenza viruses, have also been observed by EM (Falk *et al.*, 1997). Immunofluorescence studies of ISAV infected cells have shown that the ISAV is present in the nucleus early in the infectious cycle, while later it is found mainly in the cytoplasm (Aspehaug *et al.*, 2004). The latter compartmentalization occurs at the onset of the production of the late proteins. In the nucleus, NP is found to concentrate in the nucleolus. NP is the only major phosphorylated protein of the ISAV particle, a property that is thought to be associated with the active transport in and out of the nucleus.

Matrix

The matrix (M) is the most abundant protein in the virion; it is a 22–24 kDa non-glycosylated protein encoded by the smaller ORF1 of genomic segment 8 (Falk *et al.*, 1997, 2004; Biering *et al.*, 2002). The ISAV matrix protein is a late viral protein that accumulates in the nucleus during the infectious cycle, but it is also present in the cytoplasm. When whole virus particles are treated with NP-40, it is found in both the soluble and pelleted fraction, indicating a tight association with both the RNP complex and the soluble proteins of the envelope (i.e. the surface glycoproteins), which is in agreement with findings reported for influenza viruses (Falk *et al.*, 2004). Subsequently, it is assumed to form a shell on the inside of the envelope lipid bilayer. The ISAV M protein is not phosphorylated and it thus differs from the matrix proteins of the influenza viruses.

Accessory proteins

Polymerases

The viral polymerase complex consists of the proteins encoded by genomic segments 1, 2 and 4, and the subunits are named PB1, PB2 and PA, respectively, based on an assumed analogy to influenza viruses where PB1 and 2 are basic proteins and PA is acidic (Krossoy *et al.*, 1999; Clouthier *et al.*, 2002; Snow *et al.*, 2003b). As mentioned above, these enzymes are important parts of the RNP complex associated with the different processes necessary to replicate the viral genome. Some of the functions of the polymerase complex of influenza viruses have also been found to be present for ISAV, which indicates that these functions are much conserved.

Like the influenza viruses, ISAV has cap-stealing activity. Heterologous 8–18 nucleotide 5′-cap structures are snatched from cellular mRNA and added to the viral mRNA molecules (Sandvik *et al.*, 2000). In this process, the ultimate 3′-nucleotide of the

vRNA is lost and thus is not a part of the mRNA (Sandvik *et al.*, 2000). Cap stealing is a process performed by orthomyxoviruses that affects the natural transcription and protein expression of the cell. The process favours the viral mRNA transcripts and is thus an important factor of the outcome of the infection. The viral mRNA is polyadenylated and this is performed by the viral polymerase complex. Polyadenylation is initiated by signal 13–14 nucleotides downstream of the 5′-end terminus of the vRNA. In each ISAV genome segment, the terminal 21–24 nucleotides contain partially self-complementary panhandle structures that are important for transcriptional regulation of viral RNA (Sandvik *et al.*, 2000). The terminal 8–9 nucleotides of each genomic segment are conserved and identical for all genomic segments. In the influenza A virus, which is another genus in the *Orthomyxoviridae*, 12–13 terminal nucleotides are conserved. The number of conserved terminal nucleotides most likely reflects the replication temperature optimum for this virus, i.e. the secondary structure of the genomic segments of ISAV that replicates at a low temperature requires fewer self-complementary nucleotides for stabilization (Sandvik *et al.*, 2000).

Other viral proteins

Two mRNAs are transcribed from the ISAV genomic segment 7; one is collinear with the viral genomic RNA and the other is spliced (Biering *et al.*, 2002). The 34 kDa protein encoded from the collinear mRNA is present in infected cells but not in purified virus particles, i.e. it is a non-structural protein (Garcia-Rosado *et al.*, 2008). It has a cytoplasmic localization. The protein downregulates type I IFN promoter activity (McBeath *et al.*, 2006; Kileng *et al.*, 2007; Garcia-Rosado *et al.*, 2008). The protein is named NS, as is the non-structural IFN antagonist of influenza viruses. The function of the other viral protein encoded by segment 7, i.e. from the spliced mRNA (ORF2), is unknown (Kibenge *et al.*, 2007).

The genomic segment 8 uses a bicistronic coding strategy and encodes the matrix protein, as well as a 27 kDa protein encoded from ORF2 (Biering *et al.*, 2002; Garcia-Rosado *et al.*, 2008). The ORF2 protein is present in purified viral particles, as can be seen by Western blotting, but in a small amount as it is not observed in protein gels of purified virus. The protein binds RNA, contains two NLSs and has a mainly nuclear localization. Like the NS protein, the segment 8 ORF2 protein downregulates type I IFN promoter activity (Garcia-Rosado *et al.*, 2008).

Phylogeny and Genetic Variation of ISAV

The ISAV genome is highly conserved. The two genomic segments with the highest variability are the HE and the F. These segments are phylogenetically informative, in contrast to several of the other genomic segments where little phylogenetic information can be extracted.

Most of the phylogenetic analysis of ISAVs is based on the information obtained from the 5′-end of the *HE* gene (i.e. the 5′-end referred to is that of cDNA, it will actually be the 3′-end of the vRNA). The HPR and the inner surface region situated 3′ to the HPR are excluded because the HPR region varies through deletion and/or recombination between related isolates and is therefore of limited use as an indicator of relatedness. The 5′-end HE variation between North American and European isolates ranges between 80 and 81% at the nucleotide level, while the identities between the European isolates in the same region range from 98 to 100% (Blake *et al.*, 1999; Devold *et al.*, 2001). Variation between the European isolates in the F gene is <3% at the nucleotide level and approximately 76% between North American and European isolates (Devold *et al.*, 2006). In a full-genome analyse of 12 Norwegian isolates, the nucleotide sequence identities ranged from 89.0 to 99.7% (Markussen *et al.*, 2008). Based on the differences in the 5′-end of the HE gene, ISAV has been divided into two major clusters or genotypes, called the North American and the European, which is based on the origin of the isolates (Devold *et al.*,

2001; Nylund *et al.*, 2007). Analysis of the genomic segment 5 encoding F has supported this division (Devold *et al.*, 2006). Some ISAV isolates from North America are European-like and are often referred to as 'European-in-North America', and this group is more distant from the other European-like isolates and could perhaps be recognized as a third genotype of ISAV (Nylund *et al.*, 2007). ISAV isolates within the same outbreak cluster together in phylogenetic analysis, although they demonstrate some sequence variation. This finding indicates a common origin for isolates from the same outbreak (Lyngstad *et al.*, 2008).

The limited sequence variability of the ISAV genome probably signifies the changes that are present, and thus the European genotype can be subdivided further (Devold *et al.*, 2006; Nylund *et al.*, 2007) into different clades.

The frequency of recombination in the ISAV genome as seen in segment 5 is assumed to be higher than that of other orthomyxoviruses (Markussen *et al.*, 2008), which could indicate that a contribution of recombination in the evolution of ISAV remains elusive. Reassortment of genomic segments is found to occur for ISAV, but the frequency and importance of this remain elusive.

Inactivation of ISAV

Available data suggest that ISAV may remain infective for extended periods of time outside its host (Nylund *et al.*, 1994; Rimstad and Mjaaland, 2002). The virus is stable in the pH range 5–9, completely inactivated after 30 min at pH 4, and infectivity is reduced by 90% after 30 min at pH 11 (Falk *et al.*, 1997). Five cycles of freezing (–80°C) and thawing (20°C) do not reduce infectivity. Infectivity of tissue preparations is retained for at least 48 h at 0°C, 24 h at 10°C and 12 h at 15°C (Torgersen, 1997).

Since water is the natural environment for ISAV, attempts have been made to estimate the viral survival time in water (Rimstad and Mjaaland, 2002). Water in this context is not a constant entity but varies depending on factors such as temperature, presence of bacteria, enzymatic activities, organic material or UV radiation. The effect of these factors may not necessarily be negative for virus survival; for instance, UV radiation is effectively stopped in water, and organic material may be protective for virus survival. ISAV is an enveloped virus with glycosylated surface proteins and accordingly is attached easily to different particulate material, which could affect virus survival as well as spread. Laboratory experiments are thus limited in their ability to reproduce natural conditions and their effect on ISAV.

Published data for survival of viral infectivity are shown in Tables 4.3 and 4.4.

Transmission

The infectivity of ISAV may withstand a long time outside the host. Waterborne transmission has been demonstrated in cohabitation experiments, indicating that it is important for the spread of ISA, and then notably by horizontal spreading from nearby

Table 4.3. Survival of ISAV infectivity.[a]

	Virus in supernantant[b]		Tissue preparation[b,c]			Sterile seawater[d,e,g]		Natural seawater[d,e]		Sterile fresh water[d,e]	
Temperature	4°C	15°C	0°C	10°C	15°C	4°C	15°C	4–6°C	15°C	4°C	15°C
Survival time	14 d	10 d	48 h	24 h	12 h	105 d[f]	21 d	7 d[e]	7 d[e]	7 d	7 d

[a]The table does not describe endpoints of virus survival but states that the virus is infective at the time indicated; [b]Falk *et al.* (1997); [c]Torgersen (1997); [d]Rimstad and Mjaaland (2002); [e]MacLeod *et al.* (2003); [f]a 2-$\log_{10}$ reduction in titre after 2 weeks (MacLeod *et al.*, 2003); [g]a 3-$\log_{10}$ reduction in titre after 4 months (Rimstad and Mjaaland, 2002).

Table 4.4. Inactivation of ISAV by commonly used disinfectants.

Method	Dose	Comment	Contact time	Outcome/titre reduction	Reference
Heat	45°C		5 min	+	Torgersen (1997)
	50°C		1 min	+	Torgersen (1997)
	50°C		2 min	–	Torgersen (1997)
	55°C		1 min	–	Torgersen (1997)
	55°C		10 min	–	Krogsrud *et al.* (1991)
UVC (J/m^2)	5	Diluted ISA infective tissue homogenate		+	Torgersen (1997)
	40–100			–	Torgersen (1997)
	200			–	Torgersen (1997)
	33±3.5			3-log red.	Oye and Rimstad (2001)
	51±13	Fresh water		3-log red.	Oye and Rimstad (2001)
	72±16.31	Salt water		3-log red.	Oye and Rimstad (2001)
	1200	Waste water Processing plant effluents		–	Anon. (2000)
Formic acid	pH 3.5	0°C, H_2CO_2	8 h	–	Torgersen (1997)
	pH<3.9		24 h	–	Anon. (2000)
	pH 4.0	0°C, H_2CO_2	24 h	–	Torgersen (1997)
NaOH	pH 11.5	0°C	48 h	–	Torgersen (1997)
	pH 12	0°C	24 h	–	Torgersen (1997)
	pH 12		7 h	–	Krogsrud *et al.* (1991)
Chlorine	50 mg/ml		30 min	+	Torgersen (1997)
	100		15 min	–	Krogsrud *et al.* (1991)
Formalin	0.5%			–	Krogsrud *et al.* (1991)
	0.5%		16 h	–	Anon. (2000)
Ozone	8 mg/ml	600–750 mV redox potential	4 min	–	Anon. (2000)
Virkon S (peroxygen compound)	1:100 1:200 (in hard water)	Tested on ISAV in SHK cells	10 and 20 min at 20°C	Virucidal activity confirmed	Anon. (2000) Antec (2003)

infected farms (Thorud and Djupvik, 1988). The virus may be shed into the water by various routes, such as skin, mucus, faeces, urine and blood and waste from dead fish (Totland *et al.*, 1996). The most likely route of viral entry is through the gills, through skin injuries, the eye or through ingestion (Rimstad *et al.*, 1999). Gills being of importance as a port of entry is also indicated by the supposed non-virulent HPR0 variant that is detected mainly in the gills (Anon., 2005). In Norway, ISA is detected almost exclusively in the seawater phase, while only 0.7% of all known ISA outbreaks have been related to fish in the freshwater stage. This is probably not due to age-related resistance, as experimental infections in fresh water have demonstrated that fry and parr are susceptible to ISA, although there are indications that smolts in fresh water are more susceptible to ISA than smaller fish (Glover *et al.*, 2006).

The introduction of ISAV to a sea site without proximity to farms with recent ISA outbreaks is associated with well boats, smolt transfer, transport of infected adult fish between fish farms and release of untreated water into the sea from nearby processing plants (Vagsholm *et al.*, 1994; Jarp and Karlsen, 1997; Murray *et al.*, 2002). The sea louse (*Lepeophtheirus salmonis*) has also been suggested as a possible vector of ISAV, though the significance of this mode of transmission has never been determined (Nylund *et al.*, 1993, 1994). Whether this latter route of transmission represents a passive transfer of virus or is due to active replication of virus in the sea lice has not been clarified.

ISAV has been detected by RT-PCR in fertilized eggs from broodfish with clinical ISA (Søfteland, 2005). However, it is not known if such findings reflect that infective virus can be transmitted to the next generation through sexual products. There are no verified field observations that can confirm that ISA has been transmitted vertically; thus, experience-based evidence is not supportive of significant vertical transmission. Other studies did not find transmission of virus through fertilized eggs from ISAV-positive broodstock to the offspring, or transmission after injection of materials from fertilized eggs into parr (Melville and Griffiths, 1999). But, the ISAV found in Chilean aquaculture from 2007 onwards had nucelotide sequences highly similar to those of European ISAV, thus indicating that introduction by vertical transmission was one of several possible routes (Vike *et al.*, 2009).

The question of vertical transmission of ISAV has been, and still is, debatable (Melville and Griffiths, 1999; Nylund *et al.*, 2007; Rimstad *et al.*, 2007; Lyngstad *et al.*, 2008; Vike *et al.*, 2009). There is a general agreement that ISAV is very seldom transmitted vertically in Norwegian salmon farming. This opinion is, however, based on accumulated experience-based knowledge and not on scientifically controlled experiments. Different strains of virus may have different abilities to succeed in vertical transmission, especially when considering the potential existence of non-virulent virus strains. Some studies, based on screening (using real-time RT-PCR), found that the majority of smolt-producing sites were positive (Nylund *et al.*, 2007). The same authors concluded (based on real-time RT-PCR and genotyping examining broodfish embryos, parr, smolt and post-smolt) that the major transmission route for ISAV in Norwegian aquaculture was vertical.

Others have challenged this conclusion and a study that summarizes epidemiological information from ISA outbreaks in Norway in 2003–2005 concludes that genetic information of the virus isolates supports associations between adjacent outbreaks, i.e. consistent with horizontal transmission of the virus (Lyngstad *et al.*, 2008). They did not find evidence for vertical transmission from their information from genogrouping of the virus and relationship to smolt suppliers or broodfish companies.

The probability that ISAV can be transmitted vertically may depend on individual characteristics such as clinical status and viral titre in the parent fish, failure of the disinfection process of fertilized eggs, intracellular or extracelluar transmission and/or strain characteristics of the virus. Thus, the probability for further spread via eggs, fry or

smolts as a result of vertical transmission will depend on the efficacy of intervening management procedures such as disinfection and prophylactic treatment post-stripping. Iodophore disinfection (100 mg/l, 10 min) is normally used after fertilization of eggs, before transportation from broodfish facilities to hatcheries, and also often after arrival at hatcheries. ISAV is sensitive to iodophore disinfection (Smail *et al.*, 2004), but potential pitfalls include virus protection by egg products, virus localized inside the eggshell, or the potential for lapse in disinfection routines. Furthermore, prophylactic antifungal treatment of eggs with formaldehyde (0.01%, 10–30 min) that is used routinely many times prior to the eyed egg stage will also inactivate ISAV.

Disease Diagnosis

Disease diagnosis is based on the concurrence of clinical and pathological signs (see above), together with detection of a systemic infection with the ISA virus. As the consequences of an ISA diagnosis can be severe, the use of several virus detection methods based on different biological principles (immunological/genetic) is recommended, and ultimately the virus should also be cultured. *In situ* detection (immunohistochemistry/hybridization) of the viral infection in the blood vessel endothelium is strongly indicative of a virulent type of ISA virus. Immunohistochemistry for ISAV on both kidney and heart should be routine procedure when ISA is suspected (Fig. 4.4). The endothelium on heart valves is often found to be positive by immunohistochemistry.

The initial signs of ISA can be limited to only a few weak fish with anaemia and non-specific autopsy findings. However, anaemia in combination with histopathological findings like erythrophagocytosis and moderate interstitial kidney haemorrhage are indications of ISA. Immunohistochemistry will often be positive in the endothelium of kidney vessels at this stage. An early diagnosis can avoid further dissemination by culling of the stock.

In cases of high mortality, dead and moribund fish with severe anaemia (haematocrit < 10), ascites and macroscopically visible bleedings, especially in liver, kidney or gut, are found frequently.

Screening for ISA virus infection

While the disease diagnosis can be cross-checked using several independent methods, screening most often relies on a single test, which needs to be validated properly. In particular, performance and operating characteristics in field situations are important when healthy populations with expected low prevalence are tested. Epidemiological sensitivity, specificity and predictive values must be determined (Nerette *et al.*, 2005, 2008). In low prevalence situations, false positive tests may classify infection-free population as infected. Verification of such test results must be performed by another independent test. In essence, validation determines whether a true finding in the laboratory is also true in the field.

Random sampling is scientifically correct when determining the prevalence level of a condition. When trying to establish the infection status of a population, a systematic testing of moribund fish over time as a part of disease surveillance is the most efficient way of establishing the ISAV infection status of a population.

Pathomorphological evaluation

A cornerstone of this evaluation is a histopathological evaluation of formalin-fixed, paraffin-embedded tissue sections. The pathomorphological evaluations are supplied routinely by an evaluation of IHC-stained tissue sections for detection of ISAV.

Cell culture isolation

Diagnostic cell culture isolation of ISAV from infected fish is usually performed using either SHK-1 and/or ASK-II cell

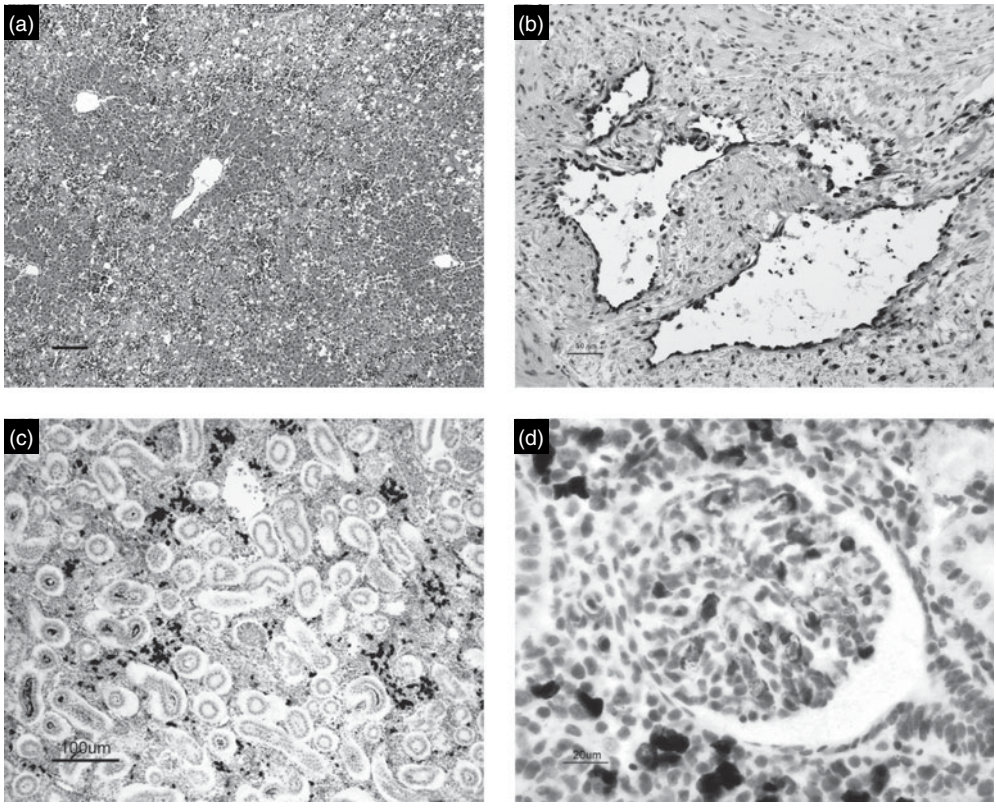


Fig. 4.4. Histological sections of Atlantic salmon with infectious salmon anaemia (ISA). (a) Liver with haemorrhagic necrosis in a zonal pattern at some distance from the central vein. In combination with severe anaemia, this change was considered originally to be a strong indication of ISA (haematoxylin-eosin stain). (b–d) Immunohistochemistry staining of ISAV giving red colour demonstrates the cell tropism of ISAV; blue colour: haematoxylin counterstain. (b) Endothelium cells of heart valves stain strongly red. (c) Staining of kidney shows strong staining of the endothelium, including the sinusoidal macrophage-like endothelium. The widespread staining demonstrates how extensive the infection can be. (d) Staining of the endothelial cells of the capillary tuft of the kidney glomerulus.

lines, but other cell lines such as CHSE-214 and TO may also support viral propagation (Dannevig *et al.*, 1995a; Devold *et al.*, 2000; Kibenge *et al.*, 2000; Wergeland and Jakobsen, 2001; Rolland *et al.*, 2005). Recent experiences indicate that ASK-II cells should be the first choice for primary isolation. ISAV in cell culture is usually identified by an immunofluorescent antibody technique (IFAT) test using anti-ISAV monoclonal antibodies (Falk *et al.*, 1998).

Demonstration of ISAV antigens

These methods include detection of ISAV using anti-ISAV antibodies on tissue cryosections (IFAT), formalin-fixed, paraffin-embedded tissue sections (IHC) and tissue imprints (IFAT) (Falk *et al.*, 1998). The methods are rapid, relatively cheap, robust and suitable for detection of ISAV in fish with clinical ISA. Detection of ISAV in subclinical infected fish is less reliable due to restricted sensitivity. IHC is currently

the first choice for detection of ISAV in diseased fish and has a major advantage in being able to associate virus detection with known target cells and pathological lesions, thus establishing a causative association between pathological and virus findings.

RT-PCR related detection

Real-time RT-PCR appears to be the method of choice to detect the presence of ISAV RNA (Mjaaland *et al*., 1997; Devold *et al*., 2000; Mikalsen *et al*., 2001; Snow *et al*., 2003a). In general, real-time RT-PCR is well suited to mass screening, since it is fast and has the capacity to detect small amounts of viral RNA, even in subclinically infected individuals. However, it should be noted that a positive test does not necessarily indicate that the fish is actively shedding virus or that the virus is virulent.

Prevention and Control

The incidence of ISA may be reduced greatly by implementation of general hygiene precautions aimed at reducing the possibility of horizontal spread and at reducing infection pressure. These include regulations related to the movement of fish, mandatory health control and transport and slaughterhouse regulations. Specific measures including restrictions on affected, suspected and neighbouring farms, enforced sanitary slaughtering, generation segregation ('all in/ all out'), as well as disinfection of offal and waste water from fish slaughterhouses and fish processing plants may also contribute to reduction of the incidence of the disease. A key point for reducing the probability of transmission is an efficient health control scheme that is able to identify ISA disease problems as early as possible to prevent spread of the agent through the production line and to reduce infection pressure (ISA contingency plan).

A significant reduction in the number of new outbreaks was reported after these regulations and procedures were introduced for Norwegian fish farming.

Currently available vaccines against ISA are inactivated whole virus grown in cell culture added to mineral oil adjuvants. Such vaccines have been used in Canada, the USA and the Faroe Islands and will be used in restricted areas of Norway. Efficacy documentation of the ISAV vaccine is based on the vaccination-challenge model in the laboratory situation. Evaluation of the efficacy of the vaccines in a field situation has not been presented so far, and has also been questioned. The vaccines currently available do not result in total clearance of virus in immunized fish and they may become carriers.

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5 Viral Diseases and Agents of Warmwater Fish

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Introduction

This chapter reviews the current literature on virus diseases affecting warmwater finfish in fresh water and the marine environment. Freshwater fish form the largest segment of world aquaculture production and a major part of the catch-fish industry in inland waters (FAO, 2007). The harvest of farmed warmwater fish is increasing, due to financial investment and refinement in technologies and a continuous increase in cultivated species. Fry production of fish species for aquaculture has also increased rapidly and disease problems have become the largest obstacle in aquaculture operations. Furthermore, some diseases of cultured fish have currently spread to wild populations of fish (e.g. koi herpesvirus disease; Haenen and Hedrick, 2006).

Wolf (1988) has described 59 fish viruses and fish viral diseases and, subsequent to that review, the number of known fish viruses has surpassed 100 and continues to increase (Fijan, 1999). Viruses of cultivated cyprinid and other warm-freshwater fish of major importance are shown in Tables 5.1 and 5.2, and viruses of cultured marine fish in warm water are presented in Table 5.3. Infectious pancreatic necrosis virus (IPNV) and lymphocystis virus are omitted and are considered elsewhere in this book. Of the viruses enumerated in the tables, this chapter focuses on spring viraemia of carp virus (SVCV), channel catfish virus (CCV, = IcHV-1), koi herpesvirus (KHV, = CyHV-3) piscine nodavirus (betanodavirus), iridoviruses including epizootic haematopoietic necrosis virus (EHNV) and red sea bream iridovirus (RSIV).

The diseases described in this chapter are transboundary aquatic animal diseases, currently of international concern due to their socio-economic importance within affected countries and their significance in the international trade of aquatic animals and aquatic animal products. Most of the diseases discussed are listed by the World Organisation for Animal Health (OIE, 2006; 2009a). Viruses causing limited infections in cultivated species, those of low virulence or causing hyperplastic lesions in feral fish and those reported only once are not discussed.

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Table 5.1. Major viruses of cultured cyprinid fishes.

Virus	Taxonomy (family)	Main host	Reference
Carp herpesvirus (CHV) (=CyHV-1)	Alloherpesviridae	Carp (*Cyprinus carpio*)	Sano *et al*. (1985)
Goldfish haematopoietic necrosis virus (GFHNV) (=CyHV-2)	Alloherpesviridae	Goldfish (*Carassius auratus*)	Jung and Miyazaki (1995)
Koi herpesvirus (KHV) (=CyHV-3)	Alloherpesviridae	Carp	Hedrick *et al*. (2000)
Spring viraemia of carp virus (SVCV)	Rhabdoviridae	Carp	Fijan *et al*. (1971)
Golden shiner virus (GSV)	Reoviridae	Golden shiner (*Notemigonus crysoleucas*)	Plumb *et al*. (1979)
Grass carp reovirus (GCRV)	Reoviridae	Grass carp (*Ctenopharyngodon idella*)	Chen and Jiang (1984)
Carp oedema virus (CEV)	Pox-like virus	Carp	Ono *et al*. (1986)

Table 5.2. Major viruses of warm-freshwater species.

Virus	Taxonomy (family)	Main host	Reference
Channel catfish virus (CCV) (=IcHV-1)	Alloherpesviridae	Channel catfish (*Ictalurus punctatus*)	Fijan *et al*. (1970)
Ictalurus melas herpesvirus (IcmHV) (=IcHV-2)	Alloherpesviridae	Black bullhead (*Ameiurus melas*)	Hedrick *et al*. (2003)
Herpesvirus anguillae (HVA) (=AngHV-1)	Alloherpesviridae	Japanese eel (*Anguilla japonica*), European eel (*A. anguilla*)	Sano *et al*. (1990)
White sturgeon herpesvirus 1 (WSHV-1) (=AciHV-1)	Alloherpesviridae	White sturgeon (*Acipenser transmontanus*)	Hedrick *et al*. (1991)
White sturgeon herpesvirus 2 (WSHV-2) (=AciHV-2)	Alloherpesviridae	White sturgeon	Watson *et al*. (1995)
Aquabirnaviruses (e.g. Eel virus European)	Birnaviridae	Various freshwater species	Sano *et al*. (1981)
Epizootic haematopoietic necrosis virus (EHNV)	Iridoviridae	Redfin perch (*Perca fluviatilis*)	Langdon and Humphrey (1987)
European catfish virus (ECV)	Iridoviridae	Black bullhead	Pozet *et al*. (1992)
European sheatfish virus (ESV)	Iridoviridae	Sheatfish (*Silurus glanis*)	Ahne *et al*. (1989)
Largemouth bass virus (LMBV)	Iridoviridae	Largemouth bass (*Micropterus salmoides*)	Plumb *et al*. (1996)
White sturgeon iridovirus (WSIV)	Iridoviridae	White sturgeon	Hedrick *et al*. (1990)
Infectious spleen and kidney necrosis virus (ISKNV)	Iridoviridae	Various freshwater species	He *et al*. (2000)
Viral haemorrhagic septicaemia virus (VHSV) (genogroup IVb)	Rhabdoviridae	Various freshwater species	Elsayed *et al*. (2006)
Pike fry rhabdovirus (PFRV)	Rhabdoviridae	Northern pike (*Esox lucius*)	de Kinkelin *et al*. (1973)
Perch rhabdovirus (PRV)	Rhabdoviridae	Redfin perch	Dorson *et al*. (1984)
Snakehead rhabdovirus (SHRV)	Rhabdoviridae	Snakehead fish (*Ophicephalus striatu*)	Kasornchandra *et al*. (1992)

Table 5.3. Major viruses of warmwater marine fish species.

Virus	Taxonomy (family)	Main host	Reference
Flounder herpesvirus (FHV)	Alloherpesviridae	Japanese flounder (*Paralichthys olivaceus*)	Iida *et al.* (1989)
Pilchard herpesvirus (PHV)	Alloherpesviridae	Pacific sardine (*Sardinops sagax*)	Hyatt *et al.* (1997)
Aquabirnaviruses (e.g. Yellowtail ascites virus [YTAV])	Birnaviridae	Yellowtail (*Seriola quinqueradiata*), Japanese flounder	Sorimachi and Hara (1985)
Lymphocystis disease virus (LCDV)	Iridoviridae	Various marine and freshwater species	Wolf *et al.* (1966)
Red sea bream iridovirus (RSIV)	Iridoviridae	Various marine and brackishwater species	Inouye *et al.* (1992)
Betanodaviruses (SJNNV, TPNNV, BFNNV, RGNNV)	Nodaviridae	Various marine species	Mori *et al.* (1992)
Viral haemorrhagic septicaemia virus (VHSV) (e.g. genogroup II, III, IVa)	Rhabdoviridae	Flatfish, cod and others	Brunson *et al.* (1989)
Hirame rhabdovirus (HIRRV)	Rhabdoviridae	Japanese flounder	Kimura *et al.* (1986)

Spring Viraemia of Carp

Introduction

Spring viraemia of carp (SVC) is an acute, systemic, contagious disease caused by a rhabdovirus (see Bootsma and Ebregt, 1983; Wolf, 1988; Ahne *et al.*, 2002). The term SVC and the name *Rhabdovirus carpio* (RVC) for the causal agent were introduced by Fijan *et al.* (1971). The infection occurs primarily in the common carp. Several other cyprinids and some other species are also susceptible. SVC typically occurs at water temperatures below 18°C and predominantly in the spring.

Prior to the description of SVC as a separate aetiological and clinical entity, this disease was part of the condition known as infectious dropsy of carp (IDC) or infectious ascites, haemorrhagic septicaemia, red contagious disease or rubella. IDC was considered initially to be a bacterial disease. The distinction of two forms of IDC, an acute or ascitic and a chronic or ulcerative form, is to some extent a matter of controversy, as mixed infections with bacteria are common in fish. The cytopathic agents from IDC described by Tomašec *et al.* (1964) and by Osadčaja (1964) might have been spring viraemia of carp virus (SVCV). Fijan (1972) indicated that IDC and its synonyms covered several distinct aetiological and pathological entities. It was clear that the acute or ascitic form of IDC encompassed SVC, and some of the chronic IDC corresponded to carp erythrodermatitis caused by atypical *Aeromonas salmonicida* (Fijan, 1972; Bootsma *et al.*, 1977; Fijan, 1999). Swim-bladder inflammation (SBI), *Pseudomonas fluorescens* infection, motile aeromonad infection and some cases of columnaris disease with skin lesions were also covered in IDC.

The disease agent

Spring viraemia of carp virus (SVCV), also known as *Rhabdovirus carpio* (RVC), is currently classified as a species in the genus *Vesiculovirus* of the family Rhabdoviridae

(order Mononegavirales) according to the ICTV (2009) (see also Hoffmann *et al.*, 2005; Kuzmin *et al.*, 2009). The aetiological role of RVC in SVC was confirmed by Baudouy (1975), Rudikov *et al.* (1975), Ahne (1977), Hill (1977), Tesarčík *et al.* (1977), Békési and Szabó (1979), Bucke and Finlay (1979) and Osadčaja and Rudenko (1981). Ahne (1973) and Bachmann and Ahne (1973, 1974) reported a rhabdovirus from carp with clinical signs of SBI, established its pathogenicity and named it SBI virus. Serological similarity with RVC was reported by Bachmann and Ahne (1974). The virus induced haemorrhage and inflammation of the swim bladder, a typical clinical sign in the initial stages of SBI. However, serological comparison by de Kinkelin and Le Berre (1974) and Hill *et al.* (1975) showed the SBI virus to be indistinguishable from RVC.

SVCV is 60–90 nm wide and 80–180 nm long, with typical bullet-shaped morphology. Virions bear a regular array of spicules on the surface (Fig. 5.1). The inner nucleocapsid has helical symmetry, consists of a linear, negative-sense and single-stranded RNA–protein complex, i.e. RNA polymerase (L)-nucleoprotein (N) and phosphoprotein (P) complex, and measures about 50 nm in diameter. The nucleocapsid is surrounded by a lipid-containing envelope [matrix protein (M)] with spikes [glycoprotein (G)]. The purified SVCV has a buoyant density of 1.195–1.200 g/ml in caesium chloride (Ahne, 1973; Bachmann and Ahne, 1973). Truncated particles, about two-thirds of the length of infective virions, represent defective, non-infectious virions (Fijan *et al.*, 1971; Bachmann and Ahne, 1973; Hill *et al.*, 1975; Bishop and Smith, 1977).

Structural proteins of SVCV are similar to those of the vesicular stomatitis virus (VSV) (Lenoir, 1973; Sokol *et al.*, 1974; Roy and Clewley, 1978a; Deuter *et al.*, 1982). These studies estimated the size of the proteins by analysis in SDS-PAGE, but recent sequence data for the complete genome of SVCV have allowed accurate predictions of the molecular weights ($\times 10^3$ Da) of the structural proteins: 238 for the L protein, 57 for the G protein, 47 for the N protein, 35 for the P protein and 25 for the M protein (Ahne *et al.*, 2002). The RNA polymerase of the virus is RNA-dependent, with optimum *in*

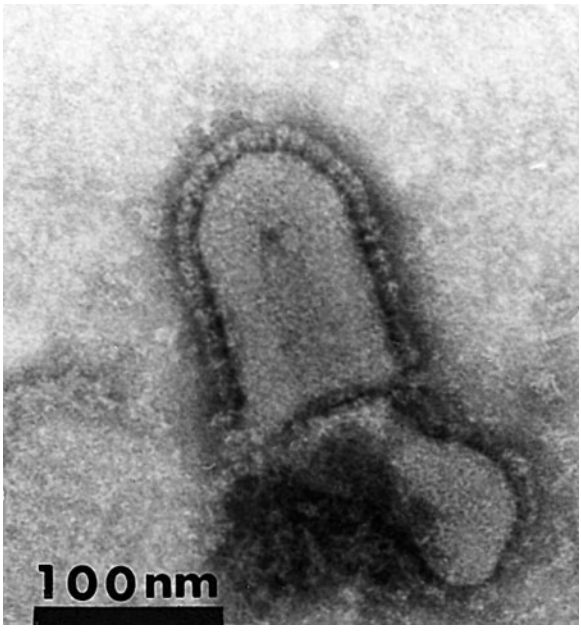


Fig. 5.1. Bullet-shaped virion of SVCV showing an array of surface spicules. Courtesy of P. de Kinkelin.

vitro enzymatic activity at 22°C. The glycoprotein on the virion's surface is responsible for its infectivity and immunogenicity (Bishop and Smith, 1977).

Antigenic studies with rabbit polyclonal neutralizing antibodies indicate that all isolates of SVCV examined belong to a single serotype. The immunochemical and biological examinations on 22 rhabdovirus isolates from fish of the Cyprinidae, Esocidae and Siluridae show that SVCV and pike fry rhabdovirus (PFRV), a rhabdovirus of the vesiculo group, share common antigenic determinants on the G, N and M proteins that prevent the two viruses being distinguished by an indirect fluorescent antibody test (IFAT). SVCV and PFRV can be differentiated by an enzyme-linked immunosorbent assay (ELISA) and by a virus neutralization test. Jørgensen *et al.* (1989) suggest that SVCV and PFRV are two serotypes of a single virus species.

Phylogenetic analysis of the partial *G* gene of SVCV and PFRV isolates identified four genogroups (I to IV), and SVCV was assigned genogroup I (Stone *et al.*, 2003) and identified as four subgenogroups (Ia–Id) that were consistent with the geographical origin. The viruses originating in Asia were assigned to subgenogroup Ia, those from Moldova, the Ukraine and Russia to Ib and Ic, and those from the UK to Id (Stone *et al.*, 2003). Isolates from the first occurrence of SVC in the USA in 2002 and in Canada in 2006 belonged to Ia (Dikkeboom *et al.*, 2004; Garver *et al.*, 2007; Warg *et al.*, 2007). Recent phylogenetic analysis shows there are at least two subgroups within Ia among isolates from the USA, the UK and China (Miller *et al.*, 2007; Zhang *et al.*, 2009) and within Id among the Austrian isolates (Basic *et al.*, 2009). The diversity of the nucleotides suggested that SVCV probably evolved independently in several different geographical areas (Stone *et al.*, 2003).

The virus replicates in a variety of fish and other vertebrate primary cell cultures and cell lines, causing a clear cytopathic effect (CPE). The best systems for viral replication are cell lines from cyprinid fish, such as *epithelioma papulosum cyprini* (EPC) (Fijan *et al.*, 1983), fathead minnow (FHM) (Gravell and Malsberger, 1965) and carp leucocyte culture (CLC) (Faisal and Ahne, 1990). Each produces high virus yields (10^8–10^9 $TCID_{50}$/ml). A number of other widely used fish cell lines, such as bluegill fry (BF-2), brown bullhead (BB) and rainbow trout gonad (RTG-2), also support replication of SVCV, but the yields are somewhat or much lower. The chicken embryo, fetal calf kidney and pig kidney cells, baby hamster kidney (BHK-21), Vero, MDCK, SK (Ahne, 1973; Bachmann and Ahne, 1974), human diploid lung (WI-38) and several reptilian cell lines (Clark and Soriano, 1974) are also susceptible to SVCV if incubated at 20–22°C.

Replication in cell cultures takes place between 4°C and 32°C, with the optimum at 20–22°C. The CPE is characterized by rounding, detachment and lysis of cells (Fig. 5.2). Margination of nuclear chromatin and cytoplasmic vacuolation precede rounding of cells and can be seen in fixed and stained cell sheets. FHM cells incubated at 20°C synthesize first progeny virus 4–6 h after infection and peak titres of both cell-associated and cell-free virus are reached between 10 and 22 h. One growth cycle lasts 8–10 h (Bachmann and Ahne, 1974; de Kinkelin and Le Berre, 1974). Well-defined plaques are formed after 3 days at 20°C.

The virulence of individual isolates may vary and can be specifically higher for the species or age of fish from which the virus was isolated (Ahne, 1986; Shchelkunov and Shchelkunova, 1989); however, this can be reduced by passage in mammalian and fish cell lines (Fijan *et al.*, 1977b; Kölbl, 1980).

Geographical distribution and host range

SVC was considered to be limited to most European countries and certainly to the western independent states of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine). Subsequently, SVC was recorded in the USA in 2002 (Goodwin, 2002; Dikkeboom *et al.*, 2004), and presence of the virus in carp was

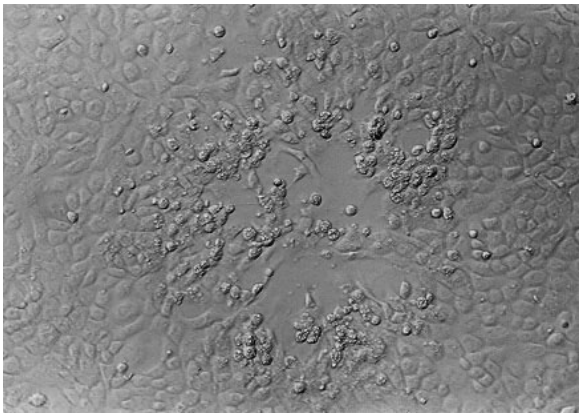


Fig. 5.2. Cytopathic effect of SVCV in EPC cells. Rounding up and detachment of cells in a focal area. ×230.

confirmed in the People's Republic of China in 2004 (Liu *et al.*, 2004). The first detection of SVCV in wild common carp from Lake Ontario, Canada, was reported in 2006 (Garver *et al.*, 2007).

Carp is the main species affected by SVC (Fijan *et al.*, 1971). All four scale-distribution varieties as well as coloured koi carp can be infected. Hill (1977) found that feral carp were more susceptible to SVC than farmed carp. Other species from which naturally SVCV infections have been recorded are crucian carp (*Carassius carassius*) (Kölbl, 1975), sheatfish, also known as European catfish or wels (*Silurus glanis*) (Fijan *et al.*, 1984), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*) (Shchelkunov and Shchelkunova, 1989), grass carp (=white amur) (*Ctenopharyngodon idella*) (Rudikov, 1980), goldfish (*C. auratus*) (Ahne, 1973), orfe (*Leuciscus idus*), tench (*Tinca tinca*) and rainbow trout (*Oncorhynchus mykiss*) (Svetlana *et al.*, 2006). Other cyprinid species, including roach (*Rutilus rutilus*) (Haenen and Davidse, 1993) and zebrafish (*Danio rerio*) (Sanders *et al.*, 2003), have been shown to be susceptible to SVCV by experimental bath infection. So it is reasonable to assume that other cyprinid species in temperate waters may be susceptible to infection. Some other species can also be infected experimentally, e.g. northern pike (*Esox lucius*), guppy (*Lebistes reticulatus*) and pumpkinseed (*Lepomis gibbosus*).

Economic importance of the disease

SVCV can induce serious mortality and economic losses, which fluctuate from year to year. These irregularities in epizootic patterns are poorly understood. Mortality is usually around 30%, but may exceed 70%. Most outbreaks occur after stocking of ponds in the spring. Ghittino *et al.* (1980) estimated the loss of 1-year-old carp in Europe to be 4000 t annually, i.e. 10–15% of this age group. The loss of 2-year-old carp and their replacement for restocking increases the cost of production. Since outbreaks of SVC have been less frequent in European countries during the past 25 years, currently Directive 2008/53/EC as an amendment of Directive 2006/88/EC has decided to remove SVC from the EU disease list. However, because of the economic impact of SVC, EU countries free from SVC or controlling the disease continue to organize measures related to biosecurity.

Diagnostic methods

Mortality of fingerlings and older fish can be expected at temperatures below 18–20°C, i.e. in the spring and at the beginning of the summer, but less frequently in the autumn and winter. Clinical diagnosis is based on behavioural and external and internal signs. The disease signs outlined below are based

on uncomplicated natural SVC outbreaks and experimental infections in the laboratory (Fijan *et al*., 1971; Fijan, 1972, 1973, 1975; Bachmann and Ahne, 1973, 1974). Fish in the initial stages of disease congregate near pond banks and terminally are sluggish, swim on their side, move slowly or rest in a normal or abnormal position. External signs of SVC include skin darkening, swollen belly, exophthalmos, petechial haemorrhage and ecchymoses in the skin, gills and anterior eye chamber, anaemia and pale gills, as well as protrusion and inflammation of the vent (Figs 5.3 and 5.4). Shedding of long, white to yellowish and thick mucoid casts from the vent is readily visible in fish kept in experimental facilities. Internal signs are dominated by oedema in all organs, haemorrhage, peritonitis and catarrhal enteritis. Small to large quantities of a transparent fluid in the peritoneal cavity may be tinged by haemorrhage. Fibrinous peritonitis causes organs to adhere to each other and to the parietal peritoneum. Petechial haemorrhage is evident in the internal organs and muscles. Haemorrhage is easily recognizable in the lamina epithelialis of the swim bladder. The spleen is enlarged and sometimes affected by haemorrhagic or ischaemic infarcts.

Specific diagnosis of SVC is based generally on the isolation of SVCV in cell culture, followed by its immunological or nucleic acid-based identification, such as PCR (OIE, 2006). Isolation of the virus in cell culture is the most sensitive and widely used approach. Identification of SVCV antigen in tissues of moribund and dead infected carp is possible using IFAT (Faisal and Ahne, 1984) and ELISA on tissue extracts (Way, 1991; Rodak *et al*., 1993). PCR-based methods are preferred for SVC diagnosis because of their high specificity and convenience. A reverse transcription (RT)-PCR protocol described by OIE (2006) can amplify the partial *G* gene of both SVCV and PFRV and, therefore, subsequent sequence of the amplicon has to be carried out for identification. Furthermore, the SVCV subgroup can be identified using a BLAST search of the sequence data.

Isolation of SVCV from diseased fish is accomplished readily by inoculation to EPC or FHM cultures with tenfold dilutions of

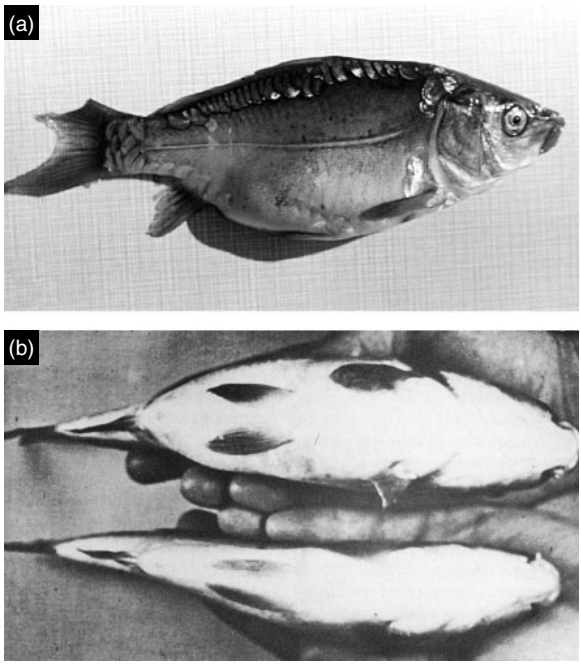


Fig. 5.3. Spring viraemia of carp. (a) Lateral view of carp showing distended abdomen and haemorrhage in skin and in anal and caudal fin. (b) Ventral view of gross abdominal distension in experimentally infected carp (top) compared with control fish (bottom).

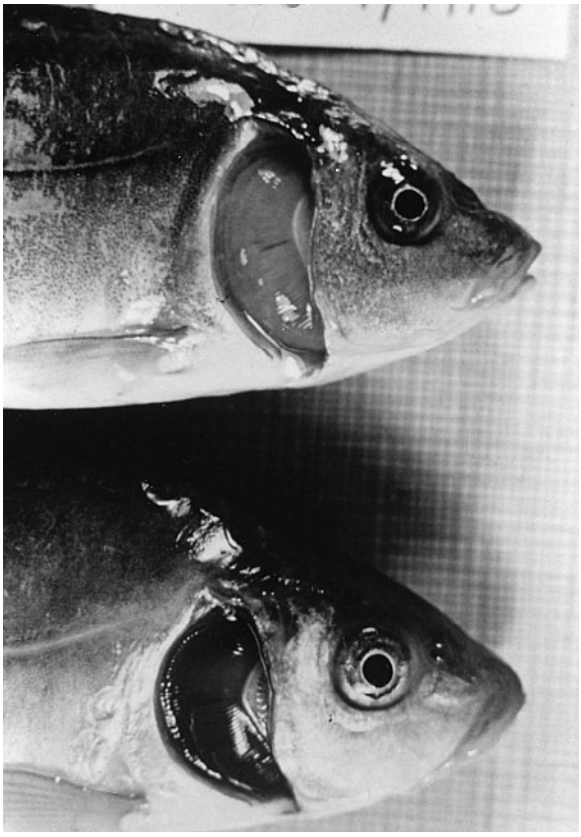


Fig. 5.4. Spring viraemia of carp. Pale gills with haemorrhage in experimentally infected upper carp compared with gills in control fish below.

homogenates of kidney, spleen, liver and encephalon (OIE, 2006). Cultures inoculated with sample should be incubated at 20°C and monitored daily for 7 days. The CPE appears as areas of cell rounding spread over the sheet, and finally all cells are detached and lysed. Samples that remain negative for CPE for 7 days should be subcultured.

Identification of isolated virus is carried out using serological techniques, i.e. virus neutralization (Petrinec, 1973; de Kinkelin and Le Berre, 1974; Fijan, 1976), IFAT (Faisal and Ahne, 1983, 1984; Rodak *et al.*, 1993), ELISA (Dixon and Hill, 1984; Way, 1991; Rodak *et al.*, 1993; OIE, 2006) or immunoperoxidase (Faisal and Ahne, 1980, 1983, 1984; Rodak *et al.*, 1993). Nucleotide-based methods are also available for virus identification: RT-PCR (Koutná *et al.*, 2003; Stone *et al.*, 2003), hybridization with RT-PCR product (Oreshkova *et al.*, 1995; Sheppard *et al.*, 2007), real-time RT-PCR (Liu *et al.*, 2008a; Yue *et al.*, 2008; Zhang *et al.*, 2009) or loop-mediated isothermal amplification (LAMP) (Liu *et al.*, 2008b; Shivappa *et al.*, 2008). SVCV is serologically distinct from all other fish rhabdoviruses, including PFRV. Some commercial identification kits are available and some of them have been compared and validated in sensitivity and specificity (Ariel and Olesen, 2001; Dixon and Longshaw, 2005). Virus neutralization with selected antisera is specific to SVCV, if complement is excluded in a virus neutralization test (Clerx *et al.*, 1978; Jørgensen *et al.*, 1989). Recently, neutralizing monoclonal antibody to SVCV has been reported (Chen *et al.*, 2008). However, some SVCV subgroups may not be completely neutralized by the monoclonal antibody. Where neutralization by antibodies to SVCV is

absent or incomplete, confirmation by RT-PCR and sequence analysis of the PCR products is recommended for the presence of SVCV (OIE, 2006).

Attempts to isolate the virus from survivors in SVC outbreaks and suspected carrier fish have been mostly unsuccessful. The only case of carp infected subclinically with SVCV was reported by Békési and Csontos (1985). They examined reproductive products of carp broodstock that were induced to spawn by hypophysation and recovered virus from 3 of 491 samples of ovarian fluid but not from all 211 samples of seminal fluid. Fijan (1988) reported negative findings in 86 samples of seminal and ovarian fluids. Ahne (1983) could not find virus in seminal fluid of experimentally infected carp. Wolf (1988) recommended using immunosuppression or low-temperature stress on broodstock and subsequent examination for the presence of virus in ovarian fluid, urine and leucocytes by co-cultivation.

Some studies have mentioned the possibility of serodiagnosis in SVC infection by demonstrating virus-specific neutralizing antibodies in the serum of fish (Sulimanović, 1973; Kölbl and Kainz, 1977; Wizigmann *et al.*, 1980, 1983; Dixon *et al.*, 1994). However, due to insufficient knowledge on the serological responses of fish to virus infections, the detection of specific antibodies to viruses has not been accepted thus far as a routine screening method for assessing the viral status of fish populations (OIE, 2006).

Genome structure and transcription

The SVCV virion contains a linear, negative-sense and single-stranded RNA of which sedimentation coefficient is 38–40 S in sucrose solution (Hill *et al.*, 1975; Lenoir and de Kinkelin, 1975; Clerx and Horzinek, 1978; Roy and Clewley, 1978b; Wu *et al.*, 1987). In the early stage of investigation on the genomic RNA, Kiuchi and Roy (1984) and Roy *et al.* (1984) defined the *M* gene sequence and a 70-nucleotide sequence at the 3′ genome terminus. The partial *L* gene, complete *G* gene and gene junction sequences were determined in 1995 and 1996 (Björklund *et al.*, 1995, 1996). In 2001, two complete genomic RNA sequences of Fijan strain (ATCC VR-1390) were submitted to GenBank by independent laboratories (GenBank Accession No. U18101 and AJ318079) and, subsequently, Teng *et al.* (2007) and Zhang *et al.* (2009) reported complete genome sequence of Chinese isolates (DQ491000; EU177782). With these data, the genome RNA of SVCV is around 11,000 nucleotide bases (Fijan strain: 11,019 bases) in length and contains five major open reading frames (ORFs), encoding five structural proteins, in the order 3′-N-P-M-G-L-5′. Each ORF in nucleotide length can be estimated as 1254 for N, 927 for P, 669 for M, 1527 for G and 6285 for L in the genome of the Fijan strain (Ahne *et al.*, 2002). SVCV genome lacks the non-virion (*NV*) gene between the *G* and *L* genes, which exists in fish rhabdoviruses of the genus *Novirhabdovirus* (Kurath *et al.*, 1997; Kuzmin *et al.*, 2009). Similarly, it does not have a large non-coding region between the *G* and *L* gene ORFs, as is found in lyssavirus genomes. Thus, the overall genome structure of SVCV is similar to that of the *Vesiculovirus* genus (Hoffmann *et al.*, 2005). Also, phylogenetic analyses using the full-length amino acid sequences of the N, P, M and G proteins, as well as partial sequences of the L proteins, confirm the close relationship between SVCV and the established members of the *Vesiculovirus* genus (Björklund *et al.*, 1996; Johansson, 2001; Ahne *et al.*, 2002). Molecular genome structure and transcription manner are reviewed by Ahne *et al.* (2002).

Pathogenesis and immunity

Ahne (1977, 1978) found that intraperitoneal (i.p.) injection caused a higher mortality rate (90%) in carp than immersion to the virus (20%) at 13°C. Intracerebral and intrapneumatic (by the swim bladder) injection of virus also induced disease, but less frequently than i.p. injection. The

introduction of virus directly into the anterior gut did not induce disease (Varović and Fijan, 1973).

Multiplication of SVCV in capillary endothelium, as well as in haematopoietic and excretory kidney tissue, causes an impaired salt-water balance, which is often lethal. Characteristic macroscopic lesions of SVC include oedema, haemorrhage, anaemia, enteritis and peritonitis (Fijan *et al.*, 1971; Bachmann and Ahne, 1973, 1974; Fijan, 1972, 1973, 1975). Histopathological changes in some organs of experimentally infected carp were described by Negele (1977). Blood vessels in the liver show a varying degree of oedematous perivasculitis, leading to necrosis. The liver parenchyma is hyperaemic, with multiple focal necroses and degeneration. The pancreas is inflamed and shows multifocal necrosis. The hyperaemic spleen shows hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres. In the kidney, both the excretory and haematopoietic tissue are damaged. Tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The peritoneum is inflamed and the lymph vessels are filled with detritus and macrophages. Changes in the intestine are dominated by perivascular inflammation, desquamation of the epithelium and atrophy of the villi. Figure 5.5a shows the intestine of a moribund carp. In the swim bladder, the epithelial lamina changes from a monolayer into a discontinuous multilayer. The heart is affected by pericarditis and by infiltration of the myocardium, which is followed by focal degeneration and necrosis. Osadčaja and Rudenko (1981) found similar changes in both natural and experimentally induced SVC. They also noted encephalitis, haemorrhage and inflammation in the spleen, as well as acute catarrhal enteritis, with necrosis and desquamation of the epithelium. Sulimanović *et al.* (1986) encountered two types of severe changes in the premortal stage of experimentally infected carp: acute, necrotic alterations with weak inflammatory reactions (Fig. 5.5b and c) or prolonged host response with hydrops and pronounced inflammation (Fig. 5.5d). Perivascular inflammation, endocarditis and lesions in the myocardium are illustrated in Fig. 5.6.

As in almost all fish diseases, temperature is the main factor determining the course and outcome of SVCV infection. Under experimental conditions, high mortality occurs within 10–15 days at 16–17°C, but later at 11–15°C (Fijan *et al.*, 1971). The disease does not develop in infected fish kept at 20–22°C or higher (Fijan, 1976) or at a constant temperature of 18°C (Baudouy *et al.*, 1980a,b,c) or 20°C (Ahne, 1980). The development of SVC occurred in carp younger than 1 month old or sheatfish under laboratory conditions at 23°C (Fijan *et al.*, 1984; Pasco *et al.*, 1987). In several long-term laboratory experiments, Baudouy *et al.* (1980a,b,c) investigated the influence of increasing and decreasing temperature on the infection. A gradual increase from 11 to 16°C will lead to faster disease development than a decrease in temperature. The gradual decrease of temperature from 11 to 5°C for 60 days, followed by a slow increase to 20°C during the next 140 days led to low mortality during the decreasing temperature and massive mortality during increase from 7 to 14°C. These findings are in agreement with field observations about the predominant occurrence of SVC outbreaks in the spring. Thus, SVC can be expected from November to July, with a peak in April–June (Fijan, 1988).

Uptake and multiplication of SVCV in carp was studied by Ahne (1977, 1978) at 13°C. After infection by immersion, the virus was detectable in gills up to 2 h and then on the fourth day, when it was also present in kidney, liver, spleen and gut. Viraemia was evident on day 5 and reached 5×10^7 plaque-forming units (pfu)/ml on day 6. Virus was detected in faeces on day 11. At 11°C, Baudouy *et al.* (1980c) found the virus in gills and viraemia on day 2 and by day 3, respectively. High viral titres in kidney, liver and spleen of naturally and experimentally infected carp (Fijan *et al.*, 1971; Ahne, 1977, 1978) indicate that these organs are primary targets of virus replication. Twenty-four days after infection, the kidney has a much lower (or none) virus titre than the liver and spleen (Faisal and Ahne, 1984).

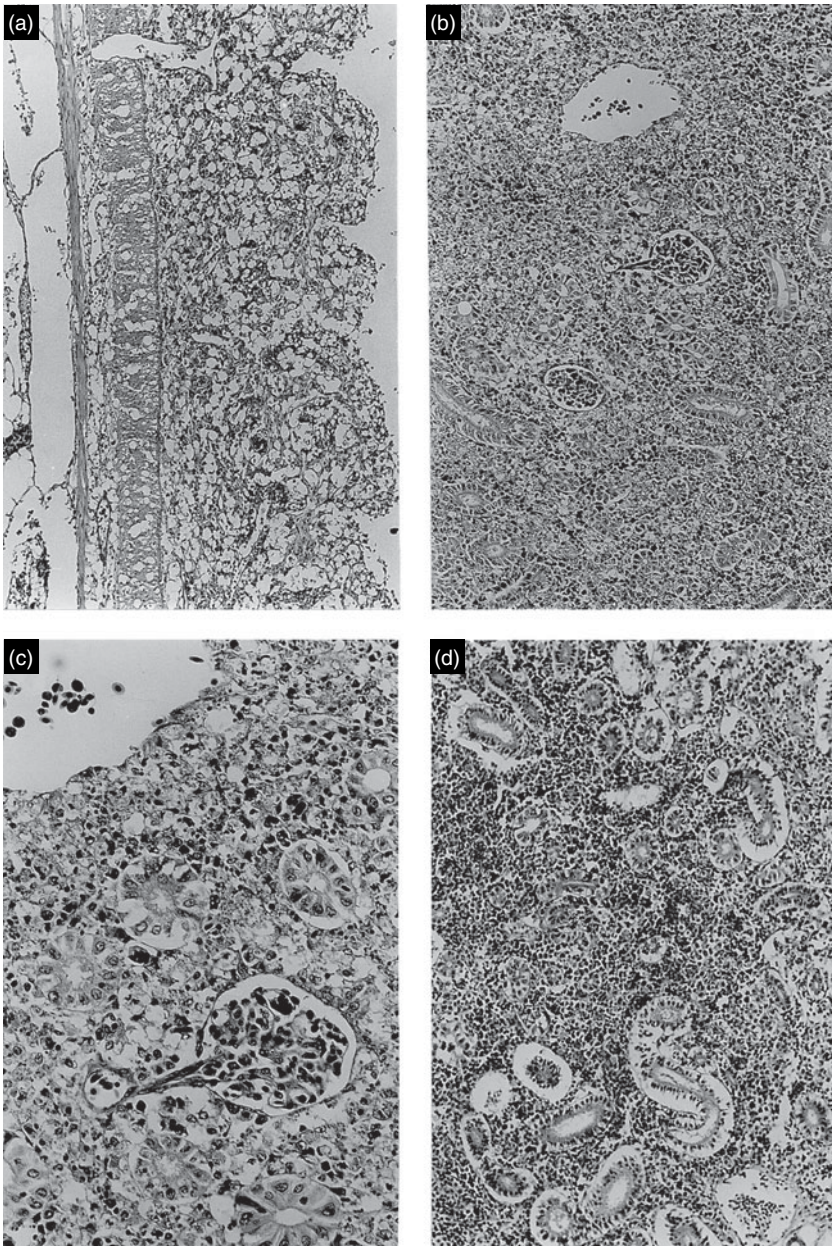


Fig. 5.5. Histopathology of the moribund stage in experimentally induced SVC. Courtesy of M. Sulimanovic and T. Miyazaki. Haematoxylin and eosin (H & E). (a) Intestine: necrosis and sloughing off of the epithelial layer; infiltration, oedema and necrosis in submucosa; oedema in the internal muscular layer; oedema and infiltration between internal and external muscular layer and in visceral peritoneum. ×80. (b) Trunk kidney: diffuse necrosis of haematopoietic tissue. ×100. (c) Detail from (b): almost all cells of haematopoietic tissue are necrotic; a varying degree of cell degeneration and some necrosis in tubuli. ×250. (d) Trunk kidney: focal infiltration in haematopoietic tissue; peritubular oedema, partial degeneration and necrosis in tubuli. ×120.

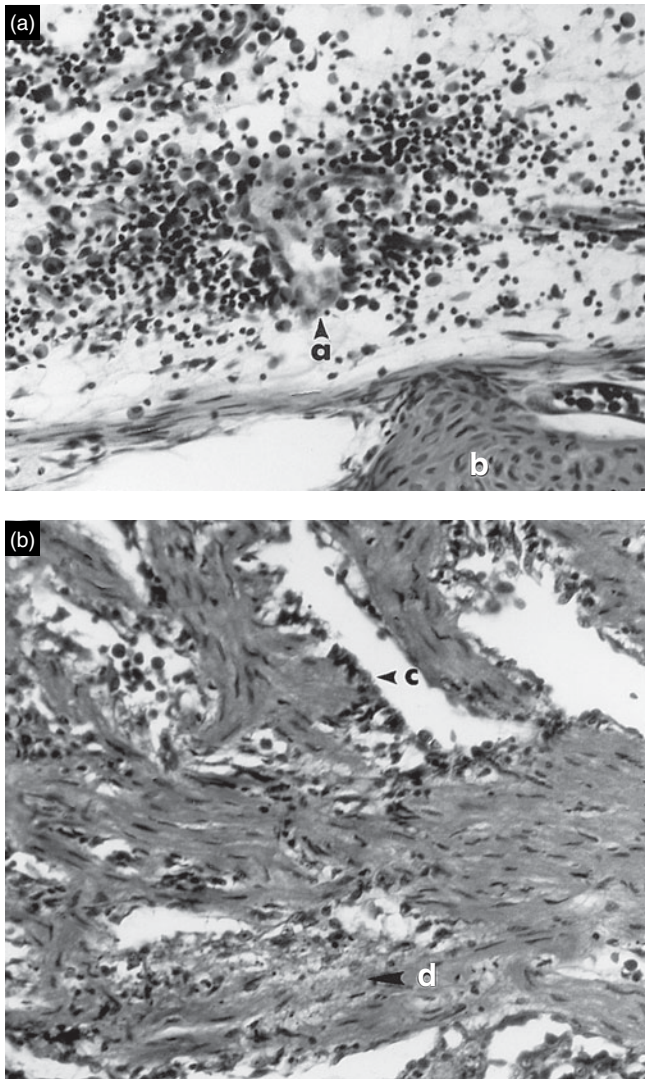


Fig. 5.6. Histological sections of gills and heart of carp with signs of SVC 7 days after experimental infection. (a) Degeneration of a small blood vessel [a] near the gill arch, surrounded by inflammation in oedematous loose connective tissue; [b], cartilage. (b) Ventricle of the heart. Endocarditis [c], focal degeneration and necrosis in myocardium [d]. H & E.×180.

The defence system has an important role in the pathogenesis of SVC and encompasses neutralizing antibodies, resistance to reinfection (Fijan *et al.*, 1977a,b; Hill, 1977; Ahne, 1980; Baudouy *et al.*, 1980b) and interferon synthesis (Baudouy *et al.*, 1977; de Kinkelin *et al.*, 1982). Little is known about the cellular immune response that is involved in protective immunity in fish without neutralizing antibodies. Temperature has a decisive influence on the carp–SVCV interactions. The virus replicates *in vitro* at a wide temperature range, with an optimum at 20–22°C, but the defence system

of immunologically mature fish restricts this activity *in vivo* to suboptimal temperature. Viraemia, detectable virus shedding and mortality occur mainly below 15°C, when carp are not capable of rapid interferon and neutralizing antibody synthesis (Fijan, 1988). After i.p. injection of the virus, the incubation period and survival times at 16–17°C are 3–7 days and 8–10 days, respectively (Fijan *et al.*, 1971). Incubation after infection by immersion lasts 7–15 days at 16–17°C and is much longer below 10°C (Baudouy *et al.*, 1980c). Production of antibodies against SVCV is influenced by the age and condition of the fish, route of infection and, most importantly, temperature. Intraperitoneally injected adult carp produce neutralizing antibodies slowly and unevenly at about 14°C, but at 25°C production is consistent, faster and with higher titres (Fijan *et al.*, 1977a). Most fish that survive infection at 13°C have peak neutralizing antibody titres after 2–2.5 months, which decline at 4 months (Baudouy, 1978). Ahne (1980) found neutralizing antibodies in carp infected by immersion after 7 days at 20°C and after 7 weeks at 13°C. However, Hill (1977) did not locate measurable neutralizing antibodies in fish kept at 15°C 10 weeks after exposure by immersion, while Fijan *et al.* (1977b) detected neutralizing antibodies in only 7.5% of 118 sera collected 1 and 2 months after oral application of a live vaccine.

Control, treatment and epizootiology

Békési and Csontos (1985) found virus in about 0.6% of the examined ovarian fluids; the scarcity of natural SVC outbreaks among fry and fingerlings indicates that vertical transmission is of minor importance. SVC is generally transmitted horizontally. Gills are the natural portal of virus entry and primary replication. Overtly infected fish shed virus in faecal casts and possibly in urine and gill mucus (Pfeil-Putzien, 1977; Ahne, 1978; Pfeil-Putzien and Baath, 1978; Baudouy *et al.*, 1980a,b,c). Demonstration of a persistent viraemic phase in experimentally infected carp kept at temperatures below 13–14°C (Ahne, 1977, 1978, 1980; Baudouy *et al.*, 1980b,c) suggests that SVCV shedding from fish during winter is the most important factor in transmission. Virus shedding by survivors of infection has not been demonstrated but appears to be important for survival of virus in fish populations. Since SVCV retains infectivity for a long time in water or mud or in a dry state (Ahne, 1982a,b), the pond environment and the fishery equipment on SVCV-positive farms should be regarded as infected with the virus.

SVCV is inactivated by lipid solvents, heating (60°C for 15 min), glycerol, ozone and diethylpyrocarbonate, as well as by pH below 4 and above 10. Formalin (3%) for 5 min, sodium hydroxide (NaOH) (2%) for 10 min, chlorine (500 mg/l) for 20 min, iodine compounds (200–250 mg/l) for 30 min, benzalkonium chloride (100 mg/l) for 20 min, alkyltoluene (350 mg/l) for 20 min, chlorhexidine gluconate (100 mg/l) for 20 min, cresol (200) for 20 min, gamma irradiation (10^3 krads) for 10 min and UV irradiation (254 nm) for 10 min inactivate the virus (Ahne, 1982a,b; Kiryu *et al.*, 2007). Normal handling in the laboratory does not cause undue loss of infectivity. Serum concentrations of 2 or 5% have a pronounced protective effect on the infectivity during storage at room, 4°C and freezing temperatures, freeze–thaw cycles and lyophilization (Ahne, 1973; de Kinkelin and Le Berre, 1974). The infectivity is retained in tap water at 10°C, in mud (pH 7.4) at 4°C for 42 days, in stream water at 10°C for 14 days and after drying at 4–21°C for 21 days (Ahne, 1982a,b).

Sensitivity of SVCV to antiviral chemotherapeutic compounds has not been tested. Prophylactic measures on farms should include disinfection of eggs using iodophore treatment (Ahne and Held, 1980), regular physical and chemical disinfection of ponds, chemical disinfection of equipment, careful handling of fish to minimize stress and safe disposal of dead fish. Avoidance of crowding during winter and early spring is essential to reduce the spread of the virus. Avoidance of SVC seems feasible on small farms supplied by spring or well water and with drainage which prevents the entrance of fish from open recipient water. Eradication

by slaughter and disinfection has little chance of success on most large European carp farms fed by surface waters from streams or rivers, mainly due to the presence of SVCV in water supplies (Fijan, 1984). Outbreaks of SVC can be prevented or stopped in mature fish by raising water temperatures above 19–20°C. The resistance of carp to SVC can probably be increased by genetic selection. A long-term selection programme resulted in high resistance of the Krasnodar strain of carp (Kirpičnikov *et al.*, 1987; Wolf, 1988). Intraperitoneal injection of dsDNA may be an alternative method to prevent SVCV infection (Alikin *et al.*, 1996).

An effective and safe vaccine for prevention of SVC is not available. However, there are encouraging results in laboratory studies and pilot field trials with live attenuated and inactivated preparations, and DNA vaccine inserted with the viral *G* gene.

The protective immunity after vaccination at 19–20°C with an attenuated live virus in autumn lasted for about 9 months in both fish receiving $10^{6.2}$ $TCID_{50}$ of virus orally and those receiving $10^{5.8}$ $TCID_{50}$ by i.p. injection; only in the latter group was protection still effective at about 11 months (Fijan *et al.*, 1977b). Similar results were obtained by Kölbl (1980). Immersion of fish to the attenuated virus also provides protection against challenge by i.p. injection (Hill, 1977; Ahne, 1980; Fijan and Matašin, 1980). Kölbl (1990) orally vaccinated about 820,000, mostly 2-year-old, carp with attenuated strains in field trials over 5 years. Vaccine was delivered in the food, twice at 1-month intervals. No case of SVC developed in the immunized fish.

An inactivated vaccine was tested for several years in the former Czechoslovakia (Tesarčík *et al.*, 1984). The oil-adjuvant vaccine (approximately 10^4 $TCID_{50}$/ml) was i.p. injected into carp in spring. Resistance to SVC was obtained as early as 7 days after vaccination and was claimed to last up to 11 months, but the mechanism of protection was not explained. The inactivated vaccine of Matašin *et al.* (1980, cited in Fijan, 1988) also increased survival rates in carp field trials, but did not protect fish under controlled laboratory conditions. In addition, DNA vaccines for fish *Novirhabdovirus* VHSV and IHNV showed great efficacy against the infection (e.g. Lorenzen *et al.*, 1999; Corbeil *et al.*, 1999) and a commercial DNA vaccine for IHN in salmonid fish was licensed in 2005 in Canada. Kanellos *et al.* (2006) and Emmenegger and Kurath (2008) showed some prophylactic efficacy of DNA vaccine and inserted the *G* gene in experimental SVC infections using carp or its variety, koi.

Conclusions and recommendations for future studies

A better understanding of host–virus interactions should be achieved by studies on humoral and cellular immune response, including clarification of the point made by Wolf (1988) about the unclear meaning of a neutralizing antibody presence (freedom from SVCV or a carrier state). Effects of stress and husbandry practices on the outcome of infection and virus shedding should be studied in order to reduce losses by application of improved husbandry and production technology. Since eradication of SVC does not seem to be possible, research on vaccination and other preventive methods is a high priority. Information is needed on vaccine formulation (e.g. live, inactivated, synthetic or DNA, mono- versus polyvalent, use of adjuvants), delivery, fate and effectiveness under various conditions. DNA vaccines especially for ornamental carp and goldfish should be investigated for commercial usage to prevent transboundary spread of SVC through international trade. Genetic selection of carp, the varieties and susceptible species for resistance to SVC and other diseases probably using genetic markers should also be investigated.

Channel Catfish Virus Disease

Introduction

Channel catfish virus disease (CCVD) is an acute, communicable and highly species-specific disease of young channel catfish

(*Ictalurus punctatus*) in the USA. The causal agent is known as channel catfish virus (CCV) or ictalurid herpesvirus 1 (IcHV-1). Infection of susceptible, almost exclusively juvenile fish results in viraemia and mortality, which occasionally may reach 100%.

Historically, awareness of a viral disease affecting fry and fingerlings began when the channel catfish industry expanded in the mid-1960s. Severe hatchery mortality occurred in southern USA. Evidence for a herpesvirus aetiology and a proposed name of the disease were discussed by Fijan (1968) and Fijan *et al.* (1970). The virus was described in more detail by Wolf and Darlington (1971). Reviews are presented by Wolf (1988), Plumb (1989, 1994), Davison (1994), Kucuktas and Brady (1999) and Camus (2004).

The disease agent

The aetiological agent CCV is herpesvirus-like (Fijan *et al.*, 1970) and is named *Herpesvirus ictaluri* (Wolf and Darlington, 1971). Robin and Rodrigue (1980b) suggested the provisional designation ictalurid herpesvirus 1. Presently, CCV (IcHV-1) has been classified in the genus *Ictalurivirus* of the family *Alloherpesviridae* of the order *Herpesvirales*, which are composed of three families (Davison *et al.*, 2009; Waltzek *et al.*, 2009).

Dimensions of complete virions negatively stained (Fig. 5.7) vary between 175 and 200 nm and the average diameter of the icosahedral nucleocapsid formed with 162 capsomeres is about 100 nm. Virus synthesis takes place in the nucleus (Fig. 5.8). The genome of CCV is a linear, double-stranded DNA consisting of 134,226 bp with 77 predicted ORFs (Chousterman *et al.*, 1979; Cebrian *et al.*, 1983; Davison, 1992). Dixon and Farber (1980) and Davison and Davison (1995) identified the viral-specific 32 and 42 polypeptides by SDS-PAGE analysis on infected cells, respectively. Davison (1994) demonstrated about 50 proteins expressed by CCV. Polyclonal rabbit antibodies recognize CCV isolates as a homogeneous group in virus neutralization, IFAT and ELISA tests. On the other hand, virus neutralization

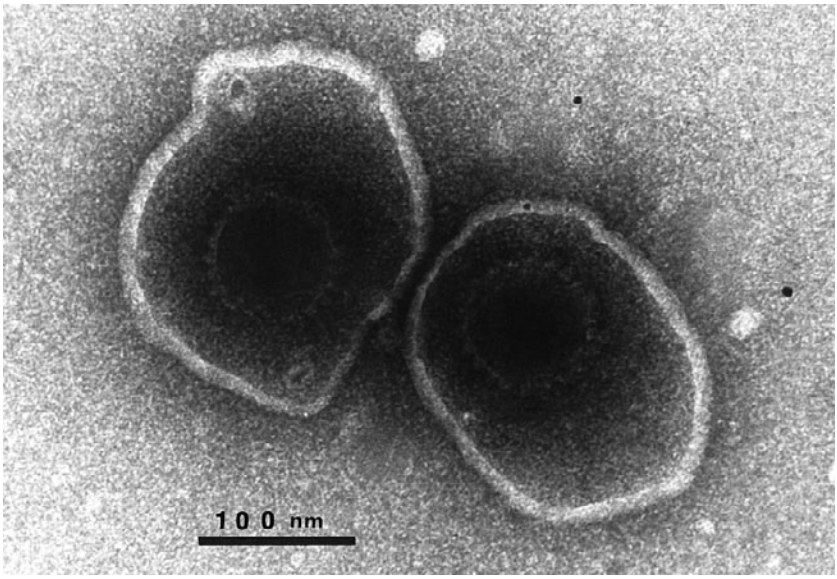


Fig. 5.7. Negatively stained virions of channel catfish virus (CCV). The membrane-bound envelope is very distinct. Nucleocapsid is located somewhat excentrically. ×298,000. Courtesy of R.C. Bird, K.E. Nusbaum and M. Toivio-Kinnucan.

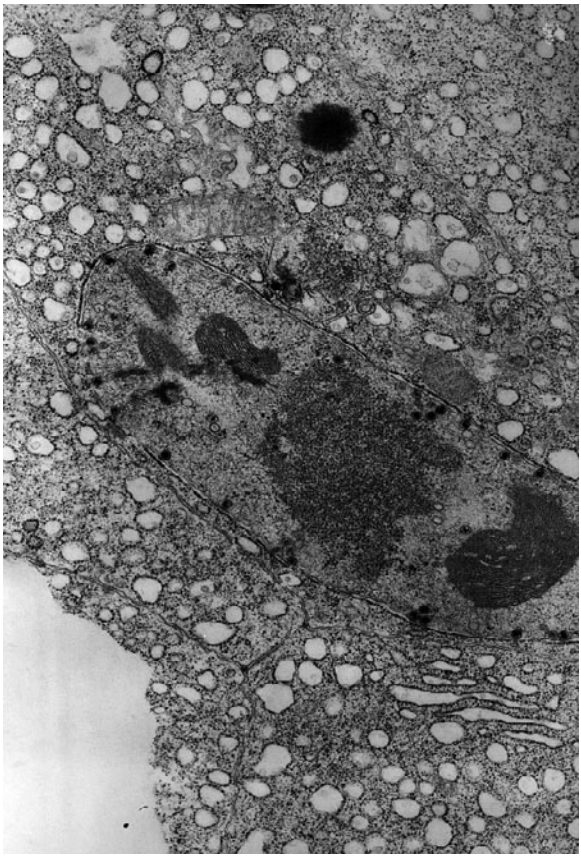


Fig. 5.8. Intranuclear channel catfish virus in spleen cell of channel catfish. The virus has migrated to the nuclear membrane, which in some places has been destroyed. Intranuclear lamellar-like inclusions are also present. ×22,000. Courtesy of J.A. Plumb.

patterns of four CCV isolates with four monoclonal antibodies indicated possible antigenic differences between strains (Arkush *et al.*, 1992). Diversity in DNA restriction digestion patterns among 12 CCV isolates was reported by Colyer *et al.* (1986).

The *in vitro* replication of CCV occurs in catfish cell cultures. These include primary cultures from channel catfish, BB (brown bullhead) cells (Fijan *et al.*, 1970), CCO (channel catfish ovary) cells (Bowser and Plumb, 1980a,b,c), continuous lines of channel catfish leucocytes (Chinchar *et al.*, 1993) and K1K cells from the more distantly related walking catfish (*C. batrachus*) (Noga and Hartmann, 1981; Walczak *et al.*, 1981). Three cell lines from marine fish (Fernandez *et al.*, 1993) are also susceptible. In stationary cultures, maximum virus yields (about 10^8 pfu/ml) are obtained in the CCO cells and the lowest ones in the K1K line and in some leucocyte lines. CCO cells show approximately 1 log greater sensitivity than BB cells at 25–33°C (Bowser and Plumb, 1980c) and a more rapid development of CPE. CCV replicates *in vitro* in the temperature range between about 10 and 35°C, with an optimum at 25–30°C. The CPE is characterized by formation of syncytia (Fig. 5.9), which are usually interconnected with cytoplasmic strands, followed by pyknosis, lysis and total destruction of the cell sheet. Changes in infected BB cultures at 30°C were monitored by Wolf and Darlington (1971). The degenerative changes started at 2 h and the first enveloped virions were completed by 10 h. These authors also described the course of virus replication and growth in BB cells. Bowser and Plumb

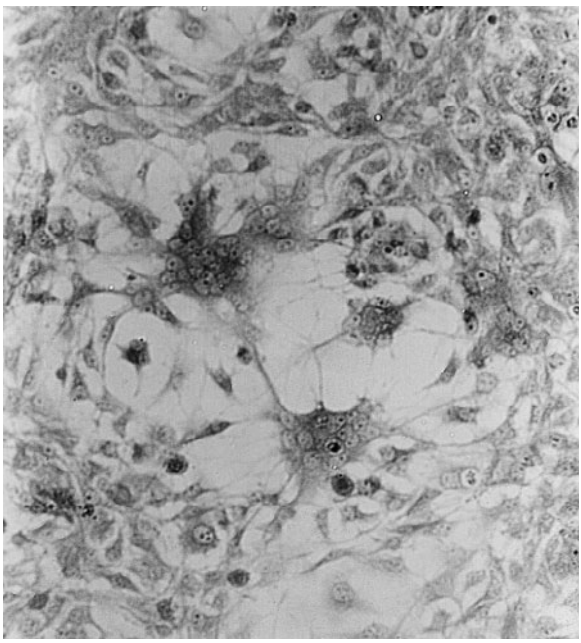


Fig. 5.9. Formation and contraction of syncytia in CCO cells about 14 h after inoculation with CCV and incubation at 30°C. Wright stain. ×230.

(1980a,b,c) studied viral growth in CCO cells, while Walczak *et al.* (1981) conducted studies in K1K cells. Somewhat more than 50% of virus remained cell-associated in BB and CCO cells, while most of it was released by K1K cells.

The virus infectivity is inactivated by ether, chloroform and glycerol. It is sensitive to acid pH, heat and UV light and is unstable in seawater (Robin and Rodrigue, 1980a). Icing or freezing at –20 or –80°C preserved infectivity for 14, 162 and 210 days, respectively (Plumb *et al.*, 1973).

Geographical distribution and host range

CCV and CCVD are endemic in reared channel catfish in most parts of the USA (Thompson *et al.*, 2005). Evidence suggests that there are infected local populations with no history of CCVD outbreaks. An outbreak of CCVD in farmed catfish was reported in Mexico in 2005 (Sánchez-Martínez *et al.*, 2007). Although a similar herpesvirus-like virus was isolated from diseased black bullhead (*Ameiurus melas*) in Italy in 1994 (Alborali *et al.*, 1996), further studies demonstrated that the virus, designated as IcmHV, was different from CCV (Hedrick *et al.*, 2003; Doszpoly *et al.*, 2008).

Natural outbreaks of CCVD occur almost exclusively in cultivated channel catfish. There is only one brief account of a natural outbreak in blue catfish (*I. furcatus*) fingerlings (Plumb, 1989). The virus has not been reported from wild channel catfish or any other wild catfish.

Experimental induction of disease by i.p. injection of virus is possible in fingerlings of blue catfish and of channel catfish × blue catfish hybrids. However, CCVD could not be induced in these fish by oral administration of the virus or by cohabitation with infected channel catfish. White catfish (*I. cams*) is susceptible to experimental infection, but disease incidence and mortality are low (Plumb, 1971a; N. Fijan, unpublished data). Fingerlings of brown bullhead (*A. nebulosiis*) and yellow bullhead (*A. natalis*) did not develop disease after injection of the virus (Plumb, 1989). Sheatfish, the African catfish (*Clarias gariepinus*),

the Asian catfish (*C. batrachiis*) and the bluegill (*L. macrochirus*) are resistant to CCV (Plumb *et al.*, 1985; Boon *et al.*, 1988; Chumnongsitathum *et al.*, 1988); the virus was detected in some specimens of these resistant fish within only a few days of infection.

Economic importance of the disease

CCVD can be economically devastating and is considered a serious problem. It has been partially responsible for the closing of at least two catfish farms in the USA and has caused decreased production in others (Plumb, 1988). Camus (2004) estimates that CCVD accounts for only 1–2% of total disease losses in catfish, although the effects on individual farms can be significant, with mortalities approaching 100% in some production units. In the Mississippi Delta, CCVD accounted for 1.8–5.8% of cases (from 1997 to 2002) received by the Aquatic Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Center in Stoneville, Mississippi. Survivors of experimental CCV infection have a retarded growth rate (McGlamery and Gratzek, 1974), but this is not obvious in natural CCVD outbreaks in commercial operations (Plumb, 1989).

Diagnostic methods

Increased mortality of channel catfish fry or fingerlings during warm weather, especially after stress, warrants examination for CCVD and sampling for laboratory tests. Hydropic conditions in fish with external signs of columnaris disease or of other bacterial infections may also be an indication of primary involvement of CCVD.

The first sign of a CCVD outbreak in fish is an increase in morbidity and mortality at temperatures above 25°C. The course of an outbreak without secondary infection is acute. Most fish die within 10 days and mortality normally ceases within 2–3 weeks. Mortality can vary from low to almost 100%, but 40–60% is common. Infected fish swim convulsively, often in spirals. In the terminal stages, they lie quietly on the bottom, respiring rapidly. Some or up to 50% of moribund fish may 'hang' head up at the water surface, but this is not pathognomonic for CCVD.

External signs of disease can vary and depend on the degree of kidney dysfunction and capillary damage resulting from the replication of CCV. Some or all of the following signs are usually present: distension of abdomen, exophthalmia, swollen and protruding vent (Fig. 5.10); haemorrhage at the base of ventral and caudal fins, in gills and skin (especially abdomen and peduncle); pale gills. Secondary infections with *Flavobacterium columnare* (=*Flexibacter columnaris*) and/or aeromonads often occur at later stages of the disease and occasionally simultaneously, causing a combination of CCVD and secondary lesions, as well as a prolonged period of mortality. At necropsy, the peritoneal cavity is hyperaemic and contains a clear, yellowish or slightly reddish fluid. Liver and kidney may be pale, with or without haemorrhage or petechiae. The spleen is congested and dark. A yellowish mucoid material, but with no food, is in the digestive tract.

The histopathology of CCVD is well documented (Wolf *et al.*, 1972; Plumb *et al.*, 1974; Major *et al.*, 1975; Plumb and Gaines, 1975). Fish with natural and experimental infections show severe changes, consisting of oedema, haemorrhage and necrosis. Kidneys are the first and the most severely affected organ. The haematopoietic tissue shows an increase in lymphoid cells, oedema, necrosis and accumulation of macrophages. Necrosis and occasional haemorrhage develop in nephrons (Fig. 5.11). The liver shows oedema, necrosis, haemorrhage and occasional eosinophilic cytoplasmic inclusions in the hepatocytes. The oedematous submucosa of the gastrointestinal tract shows multiple focal accumulation of macrophages and, in some specimens, haemorrhage. Necrosis and sloughing of intestinal lining are infrequent. The spleen is congested and its lymphoid tissue is reduced. Haemorrhage and mild necrosis are noted occasionally. Cardiac tissues may be affected by focal haemorrhage and/or necrosis.

The diagnosis of CCVD is based generally on the isolation of CCV in cell culture, followed by its immunological or nucleic acid-based identification such as PCR (OIE, 2006). Since there is no commercially available CCV-specific antiserum, and laboratory-produced antiserum to CCV is often of low titre or has cross-reaction with fish tissue, PCR is at present the most useful method in diagnosis.

Isolation of CCV from diseased fish is accomplished readily by inoculation to CCO cultures with tenfold dilutions of homogenates of kidney and spleen (OIE, 2006). The CPE may become visible after 10–12 h in the form of focal cell granulation, which is soon followed by cell enlargement and formation of syncytia. If cultures inoculated with the test material remain negative, they should be subcultivated. Formation of syncytia in CCO or BB cells is mostly specific for CCV. Titres of CCV in infected fish may reach 10^5–10^6 $TCID_{50}$ or pfu/g of tissue, but are usually lower in the early and late stages of an epizootic.

Virus identification is carried out using virus neutralization, IFAT, ELISA or a molecular-based method. The first

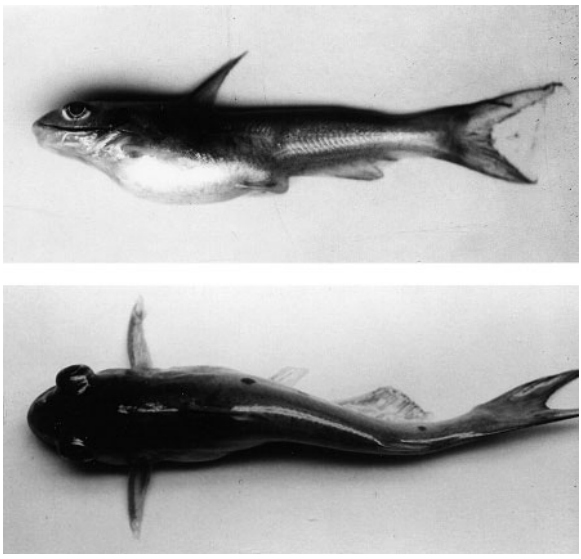


Fig. 5.10. External signs of channel catfish virus disease in fingerlings. Top: lateral view, distended abdomen and urogenital vent, bleedings at the bases of fins. Bottom: dorsal view, bilateral exophthalmia, abdominal distension and haemorrhage in anal fin.

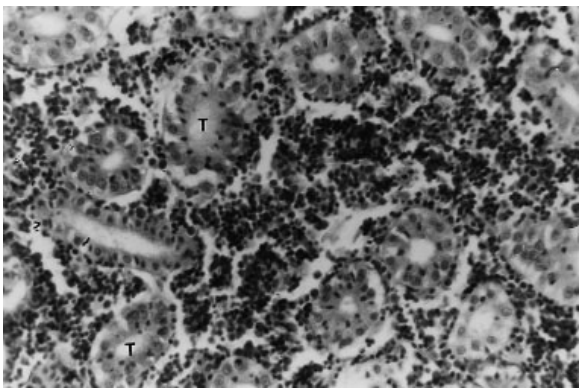


Fig. 5.11. Posterior kidney from channel catfish fingerling 3 days after injection with CCV. Large number of lymphoid cells and necrotic tubules (T). ×280. Courtesy of J.A. Plumb.

monoclonal neutralizing antibodies against CCV (Arkush *et al.*, 1992) are opening better possibilities for identification, detection and quantification of CCV. As a molecular-based method, there are several published PCR assays (Boyle and Blackwell, 1991; Baek and Boyle, 1996; Gray *et al.*, 1999) and a quantitative PCR (Kancharla and Hanson, 1996) for CCV, and OIE (2006) described a modification of the method of Boyle and Blackwell (1991).

Attempts at viral isolation from survivors of CCVD outbreaks and from suspected broodfish carriers were unsuccessful (Plumb and Jezek, 1983), until Bowser *et al.* (1985) examined a population suffering sustained mortality during the winter using co-cultivation of tissue extracts or leucocytes and blind passages of inoculated CCO cultures. Immunosuppression of broodfish by intramuscular (i.m.) injection of dexamethasone increased to 100% the viral isolation rate by co-cultivation of leucocytes. In contrast, using normal procedure and co-cultivation, Wise *et al.* (1988) could not isolate virus from stressed, non-clinically infected fry.

The first indication of the CCV antigen in broodfish ovaries was provided by Plumb *et al.* (1981) using an IFAT. Focal areas of fluorescence were recorded in spent ovaries from two immunosuppressed catfish and in the primary cell cultures from this tissue, but there was no virus replication in cultures. The authors believed the fluorescence to be a specific reaction, possibly with incomplete virus. Detection of latent CCV in clinically healthy catfish was carried out successfully using nucleic acid hybridization methods and PCR. Wise and Boyle (1985) cloned the terminal fragment of the CCV genome and used it as a specific probe for detection of viral DNA. The probe demonstrated CCV DNA in liver and other soft tissues and in erythrocytes of some channel catfish had no history of CCVD (Wise *et al.*, 1985). Bird *et al.* (1988) selected probes for detection of viral DNA expressed late in infection and one of them was highly sensitive for CCV-specific sequences. Baek and Boyle (1996) and Gray *et al.* (1999) applied the nested PCR to detect CCV DNA in latent carrier fish. Kancharla and Hanson (1996) developed a quantitative PCR which detected over 500 times more virus DNA molecules than the plaque assay of Buck and Loh (1985).

The possibility of serodiagnosis for CCVD is uncertain due to insufficient knowledge on the serological responses of fish to CCV infection, as well as other viral infections of fish. However, as direct culture of the virus or detection of viral antigen is of little use in detecting carrier fish, the identification of antibodies to CCV has more merit in screening carrier populations (Plumb, 1978; Crawford *et al.*, 1999). The antibody titres in carrier populations vary seasonally, with the lowest titres occurring in late winter and early spring (Bowser and Munson, 1986).

Genome structure and transcription

CCV genome is a linear, double-stranded DNA with 56.2% G+C nucleotide contents and has a terminal repeat sequence at both ends (Chousterman *et al.*, 1979; Cebrian *et al.*, 1983; Davison, 1992; Kucuktas and Brady, 1999). The buoyant density is about 1.715 g/ml (Goodheart and Plummer, 1975; Robin and Rodrigue, 1980b). Several authors (Chousterman *et al.*, 1979; Robin and Rodrigue, 1980b; Cebrian *et al.*, 1983) showed the molecular weight of DNA to be about 8.5×10^7 Da. Chousterman *et al.* (1979) presented details on molecular structure and restriction endonuclease sites. Dixon and Farber (1980) reported 32 virus-specific polypeptides within the molecular weight range of 12–300 kDa. Lacasa (1990) detected and characterized a protein kinase-related gene in terminal repeats of the genome. Davison (1992) sequenced the whole 134,226 bp CCV genome in the Auburn 1 strain (Accession No. NC_001493) and predicted the presence of 77 ORFs. The genome contains two identical, direct repeat regions each of 18.5 kb encoding 14 ORFs that flank the 97.1 kb unique region. CCV gene expression is regulated temporally into immediate-early (IE), early and late phases, similar to that of other herpesviruses (Dixon and Farber,

1980; Silverstein *et al.*, 1995; Huang and Hanson, 1998; Stingley and Gray, 2000), which is consistent with the kinetics of the synthesis of CCV polypeptides (Dixon and Farber, 1980). Two genes within the direct repeats, ORFs 1 and 3, expressed IE transcripts, and early RNAs were encoded by ORFs 2–9 and 11–14 (Silverstein *et al.*, 1995; Huang and Hanson, 1998; Stingley and Gray, 2000; Stingley *et al.*, 2003). ORFs 4, 7 and 10–13 expressed late transcripts after the onset of viral DNA replication (Stingley and Gray, 2000). The CCV capsid proteins, which are products of specific genes (Davison and Davison, 1995), showed no relationship to other herpesviruses in their sequences (Booy *et al.*, 1996). The thymidine kinase (TK) encoded by CCV is unique among herpesviruses and differs from the TK in the CCO host cell line (Hanson and Thune, 1993), and its transcriptional kinetics has been investigated by Silverstein *et al.* (1998). A new prospective approach with experimental annotation of the genome by proteogenomics identified 17 novel protein-coding regions (Kunec *et al.*, 2009). Kunec *et al.* (2008) reported the generation of an infectious bacterial artificial chromosome (BAC) clone of CCV, which could help mutation studies to understand the regulation and role of the pathogenesis of the genes.

Pathogenesis and immunity

CCVD occurs during warm weather (Fijan *et al.*, 1970), from May to September (Plumb, 1971a). Outbreaks are more frequent in years with high water temperatures (Plumb, 1989). Virus-injected susceptible channel catfish fingerlings suffer no or low mortality when kept at or below 15°C (Fijan, unpublished data) and the moving of infected fish from 28 to 19°C reduces mortality markedly (Plumb, 1973a). The age of the fish is also the decisive predisposing factor for outbreaks of CCVD. The disease is induced easily in susceptible channel catfish fry and fingerlings by exposure to virus in water, swabbing the gills or feeding them with virus, as well as by i.p. or i.m. injection (Fijan *et al.*, 1970). Viral injection induces disease in fish of up to 50 g (Plumb, 1971a), and occasionally the virus has been isolated from 1-year-old fish (Plumb, 1989). However, attempts to induce CCVD by exposure to virus in water have not been successful (Wolf *et al.*, 1972; McConnell and Austen, 1978) or have resulted in very low mortality in adult channel catfish (Hedrick *et al.*, 1987). Overt disease develops at an age between 2 weeks and 6 months. Natural overt infections generally are confined to fish weighing less than 10 g. Hanson *et al.* (2004) demonstrated the initial resistance of fry at 3 or 8 days posthatch (d.p.h.) and subsequent susceptibility of fry at 33–60 d.p.h. to CCVD.

The virus is most abundant in kidney, spleen, intestine and encephalon of overtly infected fish. Kancharla and Hanson (1996) also found high viral titres in the skin and gills of experimentally infected fingerlings. Radiolabelled CCV enters fish through gills and possibly also through the intestine and is concentrated in the liver (Nusbaum and Grizzle, 1987a). The kidney seems to be the primary site of viral replication, followed by other parenchymatous organs (Plumb, 1971b). A special (antiviral) class of cytotoxic cells from catfish peripheral blood leucocytes is capable of lysing virus-infected cells *in vitro* (Hogan *et al.*, 1996). Rapid viral replication in the host causes a short incubation time of about 3 days at 25–30°C and of about 10 days at 20°C. Destruction of infected cells leads to major damage of the circulatory system and of excretion, causing osmotic imbalance as well as haemorrhage. Overall, data on haematology and other clinical parameters are lacking.

The virus elicits a variable humoral immune response and resistance to reinfection. Plumb (1973b) was the first to recognize anti-CCV neutralizing antibodies in sera. Broodstock which had produced virus-infected fingerlings for 2 consecutive years had fairly constant neutralization indices throughout 1 year of examination. Gratzek *et al.* (1973) described viral neutralization in microcultures for quantitation of neutralizing antibodies. Viral neutralization activity can be found in juveniles 1 week

after i.p. and i.m. infection (Plumb, 1973b; Heartwell, 1975) and at the same time after waterborne exposure of adults (Hedrick *et al.*, 1987). Activity increases up to 9 weeks before declining. Survivors of experimental infection have anti-CCV activity in plasma 2 years after the last known exposure to virus (Hedrick *et al.*, 1987). Under natural conditions, neutralizing antibodies can be found 4 weeks after the original disease outbreak (Bowser and Munson, 1986). Neutralizing antibodies may be absent in populations which suffer a low (0.1%) mortality (Amend and McDowell, 1983). Neutralizing antibody titres vary with water temperature and are highest during summer and lowest in winter (Bowser and Munson, 1986). Continuing viral neutralization activity in sera seems to be stimulated by expression of certain viral antigens or periodic reactivation of virus (Hedrick *et al.*, 1987). Juvenile catfish can be immunized passively by injection of antiserum from adults (Hedrick and McDowell, 1987).

Hogan *et al.* (1996) described a population of cytotoxic cells among peripheral blood leucocytes that killed CCV-infected cells; even the early virus gene products rendered cells susceptible to lysis. Induction of type I interferon inhibited CCV replication in catfish cell cultures (Chinchar *et al.*, 1998; Long *et al.*, 2004) and reduced mortality in fish challenged with CCV (Plant *et al.*, 2005). Treatment of CCO cells with poly I:C inhibited significantly the expression of the CCV IE gene ORF1 in the same manner as observed in mammalian herpesviruses (Silverstein *et al.*, 2007).

Control, treatment and epizootiology

Reservoirs of virus are the overtly and the dormantly infected fish. Transmission is mostly horizontal, but the claimed vertical or 'egg-associated' transmission from carrier broodfish to eggs and fry seems to be important for viral survival and for perpetuation of infection in catfish culture.

Horizontal transmission occurs by contact and through water, and is easily demonstrable by cohabitation of virus-free catfish with infected fish. CCV is probably shed via faeces and urine. Fingerlings infected by bath and kept at 28°C shed the virus in considerable quantities from the 2nd to the 6th day (Kancharla and Hanson, 1996). Plumb (1988) assumed that fingerlings contracted the virus by cannibalizing dead or moribund fish with CCVD. The short CCV survival in pond water and mud and on tools indicates a low significance of vectorial transmission over long intervals. The role of biological vectors has not been investigated. Nothing is known about potential viral reservoirs in natural catfish populations.

Plumb and Jezek (1983) never isolated the virus from suspected broodfish. Nusbaum and Grizzle (1987b) could not demonstrate vertical transmission; yet there is circumstantial epizootiological and other evidence for vertical transmission. Outbreaks of CCVD in progeny of broodstock with high titres of CCV-neutralizing antibodies were regular (Amend and McDowell, 1984), and the viral antigen was present in the ovaries of spent females (Plumb *et al.*, 1981). The virus was isolated from broodfish in the winter (Bowser *et al.*, 1985) and the latent virus was detected in adult and in broodfish with no clinical signs, and in their apparently healthy offspring, using hybridization methods or PCR (Wise and Boyle, 1985; Wise *et al.*, 1985, 1988; Boyle and Blackwell, 1991). Egg infection, if it occurs, is accomplished by different mechanisms from those involved in the surface infection of salmonid eggs by IHN virus (Nusbaum and Grizzle, 1987b).

Approaches and methods for the control of CCVD need further refinement. Wolf (1988) suggested avoidance and the use of resistant channel catfish strains, and possibly of resistant species hybrids. Plumb (1989) considered avoidance, quarantine of infected fish populations, sanitation and optimization of hygienic (environmental) conditions in rearing facilities to be practical. Vaccination is in the experimental phase, while the manipulation of temperature is impractical and chemotherapy is unavailable.

Avoidance is the most desirable and practical approach and it can be carried out

only if virus-free stock is available. To control the spread of CCVD, Plumb (1973b) recommended avoidance of serologically positive broodstock. Amend and McDowell (1983, 1984) and Plumb (1989) warned about the limited value of serological examinations of broodstock for absence of CCV. PCR and ELISA may become crucial for broodstock screening and the reliability of the avoidance approach.

Slaughter has been recommended to prevent the spread and to eradicate the disease. Plumb (1971c) advocated the destruction both of the fish affected and of the broodstock, and the disinfection of premises and equipment. Wolf (1988) mentioned instances where affected fish were killed and ponds disinfected with 20–50 mg/l chlorine. After dissipation of chlorine and biological testing for residual CCV, ponds were stocked and the susceptible fry remained free of CCVD. Drying out of ponds should also inactivate the virus effectively. CCV is sensitive to UV irradiation (Yoshimizu *et al.*, 1989). Under simulated farm pond conditions, CCV survives less than 24 h on dried concrete chips and less than 48 h on glass cover slips or dried fishnets, and is inactivated immediately by pond mud (Plumb, 1974). The finding of Brady and Ellender (1982) that soil sediment rapidly absorbs the virus helps explain the immediate inactivation by pond mud. In pond water, the virus persists for about 2 days at 25°C and about 28 days at 4°C, but somewhat longer in dechlorinated tap water (Plumb *et al.*, 1973). It should be pointed out that the stamping-out policy could only be effective if both the water supply and the fish for restocking were free of the virus.

The reduction of water temperature to 19°C or lower decreases the mortality rate if carried out soon after infection, but not much after the onset of disease (Plumb, 1973a, 1989). Such temperature manipulation may be useful, but is rarely practical in large-scale commercial operations.

Immunization has so far received only limited attention, as economic obstacles are considerable, perhaps even prohibitive (Clem *et al.*, 1997). Progress is also hindered by the need to convince the catfish industry and potential producers of a commercial vaccine and the need for a vaccination programme (Plumb, 1989).

Although channel catfish can mount a protective immune response against CCV, 25 years have passed since the first attempts to develop a practical CCVD vaccine. The first report on experimental vaccination was presented by Noga and Hartmann (1981), who attenuated CCV by 60 *in vitro* passages in K1K cells. Intraperitoneal inoculation of attenuated virus protected the fish effectively against challenge. Adequate protection using hyperosmotic infiltration of the vaccine required two doses at a 3-week interval (Walczak *et al.*, 1981). A constructed recombinant TK gene-deletion mutant of CCV also induced protective immunity against a lethal dose of wild-type CCV (Zhang and Hanson, 1995). This CCVTK$^-$ can express foreign genes and induce antibodies against their products, thus indicating the potential of recombinant CCV for vaccine and vaccine vector development (Zhang and Hanson, 1996). Reduced shedding and persistence of this recombinant virus are also an encouragement for further research in this direction (Kancharla and Hanson, 1996). A subunit vaccine from the CCV envelope was prepared and tested in a preliminary study by Awad *et al.* (1989). Eyed eggs and 7-day-old fry were vaccinated by bath. Subgroups obtained a booster dose 2 weeks after vaccination. Vaccinated fry survived the challenge at significantly higher rates than controls. Nusbaum *et al.* (2002) reported protective immunity induced by DNA vaccine of ORF59 or ORF6 expression construct, which can elicit virus-neutralizing antibodies. A further conformational study, however, demonstrated that the published DNA vaccines failed to protect against the disease (Harbottle *et al.*, 2005).

Breeding for resistance and hybridization of channel catfish strains are a promising approach. Plumb *et al.* (1975) showed that different strains of catfish exhibited

differential resistance to CCV when the virus was mixed with their feed. A subsequent study by Plumb and Chappell (1978) examined the relative susceptibility of blue catfish (*I. furcatus*) and reciprocal blue×channel catfish hybrids to CCV. Silverstein *et al.* (2008) failed to confirm resistance of the hybrid; the blue catfish (D and B strain) were the most resistant fish (11% mortality), followed by the channel catfish from industry pool (47% mortality), the blue-channel hybrids (USDA 103×D,B strain with 63% mortality) and the channel catfish USDA 102×103 cross (68% mortality).

Although studies on stress associated with cortisol and handling did not show a relationship with mortality due to CCVD (Davis *et al.*, 2002, 2003; Small *et al.*, 2008), a response of fish to stress generally could have a substantial impact on production. Therefore, it seems that improved management practices can reduce the incidence and severity of the disease. Thune (1993) suggested proper water flow and circulation and daily removal of eggshell debris and of wasted feed, as well as feeding with nutritionally complete fry starter with at least 50% protein in hatcheries. On farms with endemic CCV, stocking densities for production of fingerlings should be below 220,000/ha and daily feeding rates below 75 kg/ha, especially during high water temperatures. Fingerlings should be harvested and handled only at temperatures below 20°C. Supplementary aeration should secure adequate levels of dissolved oxygen, especially during high water temperatures.

Conclusion and recommendations for future studies

Continued efforts to develop and test specific, sensitive, simple and practical methods for detection of latent-state CCV in fish are the prerequisite for further epizootiological studies and the control of CCVD by avoidance. The development and testing of subunit and other vaccines and exploring the potential of breeding for resistance to CCVD should be pursued.

Koi Herpesvirus Disease

Introduction

The disease agent was isolated initially from the clinical state carp and koi (ornamental coloured carp) in Israel and the USA and was named koi herpesvirus (KHV) by Hedrick *et al.* (2000). Outbreaks of a similar disease were reported earlier in Germany in 1997 (Bretzinger *et al.*, 1999; Neukirch and Kunz, 2001), and Way *et al.* (2004) found evidence of the virus as early as 1996 in their retrospective study using archival tissue samples taken during a mass mortality of common carp and koi in the UK in 1996. Subsequently, KHV has been detected in many countries, and worldwide trade of live carp has contributed greatly to the rapid spread of the disease (Haenen *et al.*, 2004; Haenen and Hedrick, 2006). This disease (KHVD) can cause mass mortality in carp and varieties in both cultured and wild environments (Haenen *et al.*, 2004) (Fig. 5.12). In 2007, the World Organisation for Animal Health (OIE) designated it as a listed disease. Valuable information on KHV is summarized in Haenen *et al.* (2004), Haenen and Hedrick (2006) and the OIE manual (2006, 2009b).

Trade of ornamental koi is quite complex compared with food fish. This makes KHV disease more difficult to control, due to the presence of carrier fish and the fact that koi are long-lived.

The disease agent

The causative agent is koi herpesvirus (KHV) (Hedrick *et al.*, 2000) or cyprinid herpesvirus 3 (CyHV-3), following the nomenclature of other cyprinid herpesviruses: CyHV-1 (carp herpesvirus [CHV], Sano *et al.*, 1985; and CyHV-2 goldfish haematopoietic necrosis

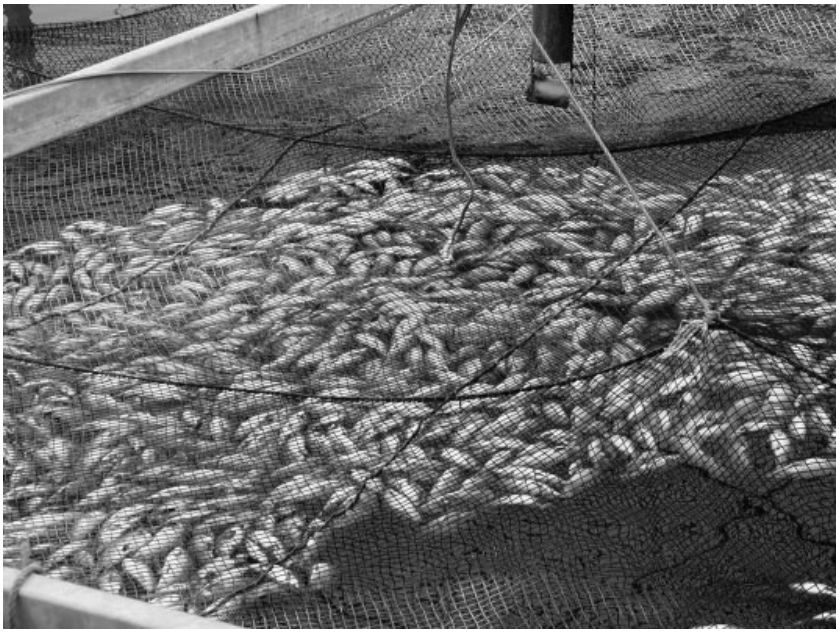


Fig. 5.12. A mass mortality due to KHV disease in net-pen cultured common carp.

virus [GFHNV], Jung and Miyazaki, 1995). CyHV-3 is currently in the genus *Ictalurivirus* of the family Alloherpesviridae of the order Herpesvirales, which are composed of three families, according to the International Classification and Taxonomy of Viruses (ICTV) (Davison *et al.*, 2009; Waltzek *et al.*, 2009). It has also been designated as carp interstitial nephritis and gill necrosis virus (CNGV) with infection with an unclassified virus (Ronen *et al.*, 2003; Hutoran *et al.*, 2005), and the evidence reported by Waltzek *et al.* (2005) supports the classification of the virus as a herpesvirus. The findings in morphogenesis of the virus in cultured cell and fish tissues provided further evidence for it to be classified in the virus family (Miwa *et al.*, 2007; Miyazaki *et al.*, 2008). Partial sequence analysis of the genome has shown that KHV is related closely to CyHV-1 and CyHV-2 (Waltzek *et al.*, 2005), and the complete genome sequence of KHV has been reported by Aoki *et al.* (2007).

Comparisons with the polypeptides, restriction enzyme digest patterns of the viral genome and DNA polymerase gene sequence on the KHV isolates obtained in different geographical regions have shown them to be identical (Gilad *et al.*, 2003; Waltzek *et al.*, 2005). More recently, it was demonstrated that isolates originating from Asian countries were distinguished from those in Europe, the USA and Israel on the partial genome sequences, which suggested that unique types of KHV were introduced independently or emerged in the respective geographic locations (Kurita *et al.*, 2009).

KHV virions possess a tegument lying between the loosely applied envelope and the nucleocapsid that gives the mature virion a diameter of 170–200 nm (Miwa *et al.*, 2007) or 183–200 nm (Miyazaki *et al.*, 2008) (Fig. 5.13). The nucleocapsid with icosadeltahedron symmetry is 110 nm (Hedrick *et al.*, 2000; Miwa *et al.*, 2007), 117 nm (Miyazaki *et al.*, 2008) and 120 nm (Hutoran *et al.*, 2005) in diameter. Miwa *et al.* (2007) demonstrated that KHV virions matured through a complex morphological pathway including budding of capsids at the inner nuclear membrane in the infected cell, as reported in other herpesviruses.

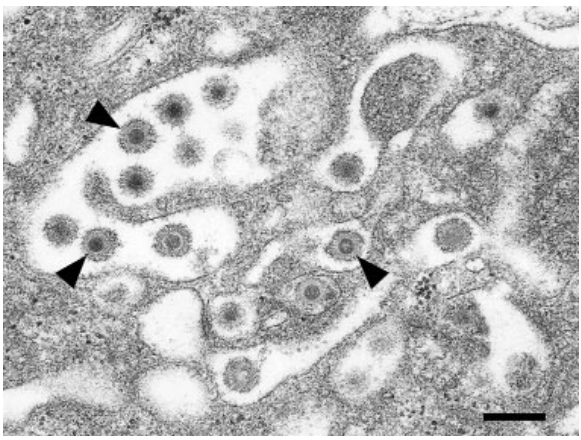


Fig. 5.13. Thin-sectioned virions (arrowheads) of KHV in cultured cells. Bar = 400 nm. Courtesy of S. Miwa.

Geographical distribution and host range

Since KHV disease was reported in Israel and the USA (Hedrick *et al.*, 2000), the virus has been spread across the globe, mostly through the worldwide trade of koi carp. Occurrence of the disease and detection of the virus have been recorded in at least 26 different countries. This includes European countries (Austria, Belgium, Czech Republic, Denmark, France, Germany, Ireland, Italy, Luxembourg, the Netherlands, Poland, Slovakia, Sweden, Switzerland and the UK) (Denham, 2003; Haenen *et al.*, 2004; Schlotfeldt, 2004; Bergmann *et al.*, 2006), Asian countries (China [Hong Kong], Chinese Taipei, Indonesia, Japan, Republic of Korea, Malaysia, Singapore and Thailand) (Choi *et al.*, 2004; Haenen *et al.*, 2004; Latiff, 2004; Sano *et al.*, 2004; Tu *et al.*, 2004; Musa *et al.*, 2005; Sunarto *et al.*, 2005), Israel (Perelberg *et al.*, 2003), South Africa (Haenen *et al.*, 2004) and the USA (Hedrick *et al.*, 2000; Gray *et al.*, 2002; Terhune *et al.*, 2004; Grimmett *et al.*, 2006).

Natural infections of KHV have only been reported in common carp and its varieties such as koi (ornamental coloured carp). Among the so-called 'common carp', there are many strains or varieties and the susceptibility to KHV varies among strains (Shapira *et al.*, 2005; Ito *et al.*, 2007a; Dixon *et al.*, 2009). The hybrids between male goldfish (*C. auratus*) and female common carp have been reported to show intermediate susceptibility to KHV, and interestingly also to CyHV-2 (GFHNV) (Hedrick *et al.*, 2006).

All age groups of carp can be susceptible clinically to KHVD. In an outbreak in net-caged carp, it was reported that mortality tended to be higher among large-sized carp compared with yearlings (Takashima *et al.*, 2005). However, small juveniles up to 1 year old were more susceptible to the disease in experimental infection trials (Perelberg *et al.*, 2005). Ito *et al.* (2007b) showed that carp larvae (3 days after hatch) were resistant to KHV infection, but the same group of carp juveniles (13 days after hatch) showed 100% mortality after exposure to KHV.

During KHV disease outbreaks in polyculture systems, mortalities have been restricted to carp and its varieties (Bretzinger *et al.*, 1999; Walster, 1999). In an outbreak in carp due to KHV infection in Lake Kasumigaura in Japan, no mortalities were observed in channel catfish, silver carp or crucian carp (*C. auratus*) which had been cultured with carp in the same cage, and a polymerase chain reaction (PCR) assay showed negative for lake smelts (*Hypomesus nipponensis*), ice fish (*Slangichthys microdon*), goby (*Tridentiger kuroiwae brevispinis*) and freshwater prawn (*Macrobrachium nipponense*) inhabiting the lake (Takashima *et al.*, 2005). Perelberg *et al.* (2003) demonstrated no clinical transmission of the disease from

KHV-infected carp to five commonly cultured fish species, tilapia (*Oreochromis niloticus*), silver perch (*Bidyanus bidyanus*), goldfish (*C. auratus*), silver carp (*H. molitrix*) and black carp (*C. idella*). Studies for transmission of the virus to goldfish, which is frequently mix-cultured with carp, showed negative results (Perelberg *et al.*, 2003; Yuasa *et al.*, 2008), but Haenen and Hedrick (2006) pointed out experimental data suggesting a susceptibility of goldfish and grass carp to KHV. El-Matbouli *et al.* (2007b) and Sadler *et al.* (2008) reported that KHV DNA was detected from goldfish cohabitated with or exposed to infected koi carp. Reverse transcription (RT)-PCR assay detecting mRNA of KHV indicating evidence of viral replicating showed negative for goldfish infected experimentally with the virus, even if the fish showed positive for viral DNA in PCR assay, suggesting that KHV in goldfish was not in active state (Yuasa *et al.*, 2008). Further studies are needed to determine whether goldfish can be persistently infected carriers or vectors in KHV disease.

Economical importance of the disease

Morbidity of affected populations of koi and common carp can be 100%, with mortality up to 90% (Haenen *et al.*, 2004). Mortality is influenced fundamentally by water temperature and this relates to the viral multiplication (Gilad *et al.*, 2003). Secondary bacterial and parasitic infections can affect the severity of the disease (Haenen *et al.*, 2004).

In aquaculture, Israel suffered great economic losses of food and ornamental carp. At the end of 1998, the loss in Israel was estimated at US$1.2 million for the common carp industry and US$0.8 million in ornamental carp exports (Perelberg *et al.*, 2003). A serial disease outbreak in Indonesia from March 2002 to December 2003 caused high mortalities (80–95%) to both koi and common carp, with estimated losses of more than US$150 million (Sunarto *et al.*, 2005). In an outbreak in carp aquaculture for food in Lake Kasumigaura in Japan in 2003, 1828 of 2148 net cages had mortalities of fish, mostly from October to November, and the dead fish reached at least 1124 t, accounting for a quarter of the annual production in the lake (Takashima *et al.*, 2005).

In a wild carp population, an outbreak of KHV occurred in Lake Biwa in Japan and the disease resulted in more than 100,000 dead fish, corresponding to 60–80% of the lake's carp population (Matsuoka, 2008). More recently, carp die-offs in Lake Mohave in May and Lake Havasu in June 2009, in Arizona, USA, were due to KHV infection.

Diagnostic methods

Infected fish swim lethargically near the surface, sometimes at the sides of a pond and gasp at the surface of the water. Some fish may exhibit loss of equilibrium, showing head-down and also 'desperate' swimming.

The most consistent gross clinical sign of disease is an irregular discoloration of the gills consistent with moderate to severe gill necrosis (Fig. 5.14). Other clinical signs include anorexia, enophthalmia (sunken eyes), fin erosion, haemorrhage on the skin and base of the fins, pale irregular patches on the skin associated with excess mucus secretion and also decrease of mucus production in patches, leaving the epidermis with a rough texture. Internal gross pathological signs are inconsistent but enlarged kidney, a swollen spleen in the early stages of the disease and a flaccid and mottled appearance of the heart have been reported (Haenen *et al.*, 2004). Gross pathologies may also be affected by infection with ectoparasites, such as *Argulus* sp., *Chilodonella* sp., *Cryptobia* sp., *Dactylogyrus* sp., *Gyrodactylus* sp., *Ichthyobodo* sp., *Ichthyophthirius* sp., *Trichodina* sp. and gill monogenean parasites, and by infection with bacteria such as *Aeromonas* sp., *Pseudomonas* sp., *Shewanella putrefaciens* and, especially, *F. columnare* at warmer temperatures (Perelberg *et al.*, 2003; Haenen *et al.*, 2004; Sano *et al.*, 2004).



Fig. 5.14. A common carp with KHV disease showing enophthalmia and necrosis of the gill filaments (arrows).

The histopathological feature of the disease is not consistent, but necrosis of gill tissues is commonly found. Hyperplasia and hypertrophy of the branchial epithelial cells may be moderate to severe, and fusion of adjacent secondary lamellae is common (Fig. 5.15). Both the enlarged branchia epithelial cells and leucocytes within the capillary spaces may have prominent nuclear swelling, margination of chromatin and pale diffuse eosinophilic inclusions. Inflammation, necrosis and nuclear margination of chromatin may be observed in other organs, the kidney, spleen, pancreas, liver, brain, gastrointestinal system, heart and skin (Hedrick *et al.*, 2000; Perelberg *et al.*, 2003; Pikarsky *et al.*, 2004; Miyazaki *et al.*, 2008).

Diagnosis is based on direct methods such as virus isolation, viral antigen detection using IFAT and target DNA amplification assay using PCR and LAMP. Among these, PCR-based assay to detect KHV genomic DNA is considered currently to be the most sensitive and reliable method and has been used preferably in diagnostic institutions worldwide.

Viral isolation can be carried out using cell lines originated from carp such as KF-1 (koi fin-1) (Hedrick *et al.*, 2000) and CCB (common carp brain) (Neukirch *et al.*, 1999), showing a characteristic CPE with syncytium formation and intense cytoplasmic vacuolation (Hedrick *et al.*, 2005) (Fig. 5.16). Other cyprinid cell lines from goldfish and silver carp show some degree of susceptibility to the virus (Davidovich *et al.*, 2007). Although FHM cell line is generally not susceptible to KHV, an unusual KHV was isolated in FHM from a case in the USA (Grimmett *et al.*, 2006). However, currently, virus isolation is not considered to be a reliable diagnostic method for KHV disease, because of the low susceptibility of the cells and rapid viral inactivation in fish tissues after death (Haenen *et al.*, 2004).

Viral antigens can be detected in imprint preparation of the affected fish tissues using IFAT with antiserum against KHV (Shapira *et al.*, 2005). KHV antigens were detected from the kidney of fish as early as 1 day after experimental exposure to the virus. This serological method is quite simple and easy to use, but it is necessary to verify the sensitivity and reliability of diagnosis using clinical samples. Currently, an ELISA method to detect KHV in fish faeces is being developed (Dishon *et al.*, 2005).

A number of PCR-based assays have been reported: as single-round PCR assay by Gilad *et al.* (2002), Gray *et al.* (2002),

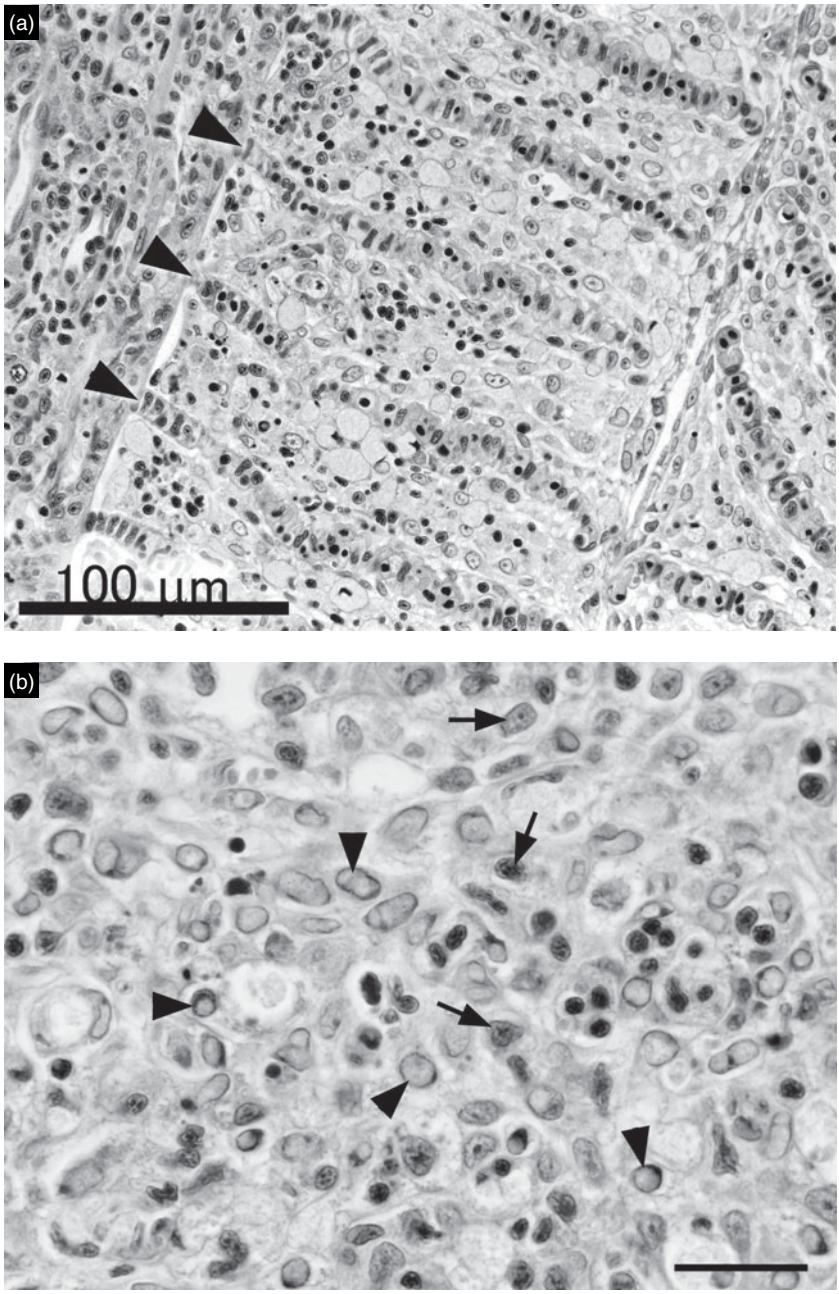


Fig. 5.15. Tissue sections of the gills of common carp infected with KHV: (a) showing fusion of secondary lamellae (arrowheads) and (b) margination of chromatin of the virus-infected cells (arrowheads) and apparently normal nuclei (arrows). H & E stain. Bar = (a) 100 µm, (b) 20 µm. Courtesy of S. Miwa.

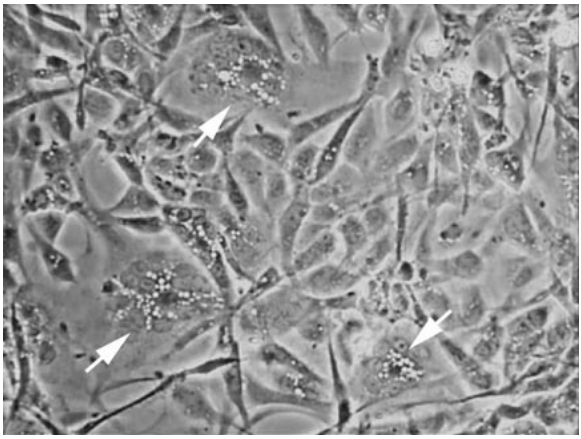


Fig. 5.16. CCB cells 5 days following infection with KHV at 20°C. Syncytium formation and cytoplasmic vacuolation are evident (arrows).

Bercovier *et al.* (2005), Ishioka *et al.* (2005) and Yuasa *et al.* (2005); as nested-PCR assay by Hutoran *et al.* (2005), Bergmann *et al.* (2006) and El-Matbouli *et al.* (2007a) and as real-time TaqMan quantitative PCR by Gilad *et al.* (2004). RT-PCR for viral mRNA detection, specifically targeting the splice viral terminase gene, has been developed by Yuasa *et al.* (2007b), but it has not been applied to diagnosis. Also, LAMP has been developed by Gunimaladevi *et al.* (2004), Soliman and El-Matbouli (2005, 2009) and Yoshino *et al.* (2006, 2009). The target organs for PCR assay are the gills, kidney and spleen, which contain abundant viral DNA, but in the case of asymptomatic survivors, the brain would be recommended to be included in the assay (Yuasa *et al.*, 2007b).

There are some limitations to the application of direct detection methods such as PCR assay to persistently or latently infected carrier fish. An alternative method to monitor surviving fish for long periods is required, as problems with the presence of carrier fish as a source of the infection will remain in the stock. ELISA methods to detect carp antibodies against KHV have been reported, but there are cross-reactions with the serum of carp infected with CyHV-1 at low level (Ronen *et al.*, 2003; Adkison *et al.*, 2005; St-Hilaire *et al.*, 2005), although it can detect antibodies in survivors after 1 year following a natural infection (Adkison *et al.*, 2005). Taylor *et al.* (2010) used ELISA for KHV antibody detection to determine the geographic distribution and prevalence of KHV exposed fish in England and Wales.

Genome structure and transcription

A study by Hutoran *et al.* (2005) estimated the genome size of KHV to be 277 kbp. The complete genome sequence of KHV (AP008984, DQ657948 and DQ177346) reported by Aoki *et al.* (2007) revealed a 295 kbp genome containing a 22 kbp terminal direct repeat. KHV thus has the largest genome reported to date for the order *Herpesvirales*. The overall nucleotide composition is 59.2% G+C. The genome encodes 156 unique protein-coding genes, eight of which are duplicated in the terminal repeat. It contains 15 genes that have clear homologues in IcHV-1 (channel catfish virus) (Aoki *et al.*, 2007). Michel *et al.* (2010) demonstrated that the genome encoded 40 structural proteins that were classified based on bioinformatic analyses as capsid (3), envelope (13), tegument (2) and unclassified (22) structural proteins, and also indicated the potential incorporation of up to 18 distinct cellular proteins in the purified virions.

The lack of sequence identity to herpesviruses of mammals precludes prediction about the functions of most KHV genes, and

the regulation of transcription of KHV may have a similar cascade manner in other herpesviruses. Rosenkranz *et al.* (2008) identified one of the membrane proteins of KHV encoded by ORF81, which was a positional homologue of ORF59 of IcHV-1. This protein with 26 kDa appeared to be non-glycosylated. Costes *et al.* (2008) constructed infectious bacterial artificial chromosome (BAC) of KHV genome and demonstrated that disruption of the thymidine kinase locus (ORF55) induced partial attenuation in koi carp.

Pathogenesis and immunity

The majority of the mode of transmission of KHV is horizontal, mostly by direct contact of fish to fish and via water. However, egg-associated transmission (vertical transmission) cannot be excluded. The main viral entry is through the epidermis (Kiryu *et al.*, 2008; Costes *et al.*, 2009) or gill tissues (Miyazaki *et al.*, 2008), and the virus can replicate rapidly and cause systemic spread in the infected fish as early as 1 day after experimental exposure. The greatest DNA concentrations are in the gill, kidney and spleen, with virus genome equivalents consistently from 10^8 to 10^9 per 10^6 host cells, and high levels of KHV DNA are also in the mucus, liver, gut and brain (Gilad *et al.*, 2004). The virus can be shed through faeces, urine, gills and skin mucus. Experiments at 23°C showed that the infected fish transmitted the virus to naive carp 1 day after cohabitation. Infectious virus was shed continuously from infected common carp for a longer period under experimental conditions at 16°C than at 23°C or 28°C (Yuasa *et al.*, 2008).

Temperature appears to be a principal environmental factor that affects *in vivo* and *in vitro* viral replication (Gilad *et al.*, 2004; Dishon *et al.*, 2007; Yuasa *et al.*, 2008). Maximal replication of the virus in KF-1 cells appears to occur between 15 and 25°C (Gilad *et al.*, 2003). KHV induced mortalities on cultured fish occur in both spring and autumn, when water temperatures range from 16 to 28°C (Perelberg *et al.*, 2003; Pikarsky *et al.*, 2004; Sano *et al.*, 2004). The disease has caused no mortality in experimentally infected fish at 29 or 30°C (Hutoran *et al.*, 2005; Perelberg *et al.*, 2005) and at 13°C (Gilad *et al.*, 2004). Shifting virus-infected fish from 13 to 23°C resulted in the rapid onset of mortality (Gilad *et al.*, 2003). Survivors, that were reared at 12°C for 125 days with no mortality after KHV infection showed occurrence of mortalities after the water temperature was raised to 23°C (St-Hilaire *et al.*, 2005). These studies indicate the possibility that infected fish surviving at low temperatures can be reservoirs of the virus (Gilad *et al.*, 2004). Moreover, these carrier fish reared at low temperature produced antibodies to the virus (St-Hilaire *et al.*, 2007), implying the persistent infection accompanying a small amount of continuous production of viral antigens in the fish. Thus, it may be considered as the worst scenario that persistently infected fish in lower temperatures shed the virus continuously, causing spread of the virus in the population and further occurrence of mortalities in the population in the subsequent warmer season.

KHV infection induces the production of antibodies in host fish, and the survivor at the permissive temperature can be resistant to further challenge of the virus. In open waters where there had once been a severe KHV outbreak, no further substantial outbreaks have been observed, probably because a large part of the population has acquired immunity against KHV, as revealed using ELISA (Miwa, personal communication). Since passive immunity to naive fish using antiserum of survivor fish has not been successful (Adkison *et al.*, 2005), other defence mechanisms such as cell-mediated immunity are also involved in the protection against KHV disease.

It appears shifting temperatures either above or below 30 or 13°C will arrest the development of clinical signs (Ronen *et al.*, 2003; Gilad *et al.*, 2004). At the early stage of influence of the disease in carp in Israel, regimens of exposure and shifting to high water temperatures had been used in the country, largely to produce 'naturally resistant' fish.

Alternatively, using a licensed commercial vaccine has currently been prevalent in the country (Perelberg *et al.*, 2005).

Control, treatment and epizootiology

In the production of koi, survivor fish of KHV disease which are persistently or latently infected with the virus are considered to have potential risk as an infection source through worldwide trade and, therefore, koi farms should aim to produce KHV-free fish.

Aquaculture facility should avoid the supply of contaminated water and the introduction of infected fish. Spring or borehole water, or appropriately disinfected river water, can be used. Using river water without any virucidal treatments, even if occurrence of KHV has not been observed, should be avoided to minimize the risks of KHV because it is present in river water for at least 4 months before outbreaks of the disease and 3 months after the end of a mass mortality (Haramoto *et al.*, 2007; Minamoto *et al.*, 2009). For monitoring KHV in river water, virus concentration methods and KHV detection procedures from environmental water have been reported (Haramoto *et al.*, 2009; Honjo *et al.*, 2010). Taylor *et al.* (2010) reported that a high proportion of the imported fish in the UK were positive for KHV antibody and they indicated that some risk stocks of fish existed that could be sourced by the ornamental carp sector. The OIE manual (2009b) recommends that biosecurity measures should include ensuring that newly introduced fish are from disease-free sources in a season with permissive water temperature and that they should be held separately in other tanks or ponds for 3 weeks, followed by inspection for KHV with a PCR assay.

In seed production operation, it is important to use KHV-free broodstock, previously screened for antibodies to the virus. Disinfection of eggs can be achieved using iodophor. KHV has been shown to be inactivated by iodophor (200 mg/l) for 30 s at 15°C (Kasai *et al.*, 2005). KHV-free juveniles of carp or koi can be produced using appropriate iodophor disinfection to eggs in a containment facility, even if the broodstock is a survivor of the disease.

Kasai *et al.* (2005) reported that the following treatment with disinfectant was effective for inactivation at 15°C: iodophor at 200 mg/l for 20 min, benzalkonium chloride at 60 mg/l for 20 min, ethyl alcohol at 30% for 20 min and sodium hypochlorite at 200 mg/l for 30 s.

KHV can survive in water for at least 4 h at 23–25°C, but not for 21 h (Perelberg *et al.*, 2003). Shimizu *et al.* (2006) showed a significant reduction of the viral titre within 3 days in environmental water or sediment samples at 15°C. Epizootic of KHV infections in natural waters is difficult to control. However, if the susceptible fish are removed from the pond and it is left empty over 3 days above 15°C, then KHV could be eradicated.

One of the ways to reduce mortality due to KHV disease is to introduce a resistant strain of common carp for food. Differential resistance to KHV disease among different common carp strains has been observed (Shapira *et al.*, 2005; Ito *et al.*, 2007b; Dixon *et al.*, 2009). The survival rate of the most resistant and sensitive strain was 60.7 and 8.0%, respectively (Shapira *et al.*, 2005). Consequently, it may be possible to establish a carp strain highly resistant to KHV infection. The other way to reduce mortality is to shift the rearing water temperature to above 30°C (Ronen *et al.*, 2003; Gilad *et al.*, 2004), but this treatment might make the survivor virus-carrier state.

Currently, a commercial vaccine is licensed in Israel only; an attenuated virus has been used to vaccinate carp (Ronen *et al.*, 2003; Perelberg *et al.*, 2005, 2008) and the protective effect of the vaccine can last at least 280 days after bath administration. Incubation for 5 days at the viral permissive temperature (24°C) just after vaccine treatment and shifting to high temperature (31°C) can make antibody induction in the fish more rapid (Perelberg *et al.*, 2008). The vaccine has been widely used in carp farms in Israel. Yasumoto *et al.* (2006) reported a liposome-based vaccine containing inactivated KHV.

To date, there has been no report of successful chemotherapy for KHV disease with

chemical compounds such as anti-herpesviral nucleotide analogue (e.g. acyclovir).

Conclusions and recommendations for future studies

KHV has spread to many countries in the world through the worldwide trade of live carp, especially ornamental koi. Trade distribution of koi worldwide is quite complex and excellent phenotype of koi broodstock is highly valuable as carp can live a long life. The most important issue is the presence of survivor carrier fish of the virus and this makes control of the disease more difficult compared with other viral diseases in aquaculture animals for consumption having one-way distribution from farm to consumer. To ensure production of KHV-free juveniles in farms, practical biosecurity measures on farms and operations including water supply and new introduction of broodstock are essential. The reliable methods of detecting carrier or persistently infected fish, including the improvement of antibody-detection ELISA less cross-reaction with CyHV-1, should be validated and standardized for screening fish such as broodstock and shipping fish and surveillance of the disease worldwide. Further studies should also involve the development of an effective vaccine which can induce antibody response in koi different from those in natural infection. In this sense, viral nature assuming immunogenicity in fish should be identified.

We have been aware of and studied KHV disease for about 10 years and, therefore, evolution of the virus, especially in pathogenicity, will be an important field of research.

Viral Nervous Necrosis

Introduction

Viral nervous necrosis (VNN; Yoshikoshi and Inoue, 1990), also known as viral encephalopathy and retinopathy (VER; OIE, 2006), caused by piscine nodaviruses (betanodaviruses), was first described in 1990 in hatchery-reared Japanese parrotfish (*Oplegnathus fasciatus*) in Japan (Yoshikoshi and Inoue, 1990) and barramundi (Asian sea bass; *Lates calcarifer*) in Australia (Glazebrook *et al.*, 1990), and then in larval turbot (*Psetta maxima*) (Bloch *et al.*, 1991), larval European sea bass (*Dicentrarchus labrax*) (Breuil *et al.*, 1991), larval striped jack (*Pseudocaranx dentex*) (Mori *et al.*, 1992), larval/juvenile red-spotted grouper (*Epinephelus akaara*) (Mori *et al.*, 1991) and cage-cultured seven-band grouper (*E. septemfasciatus*) (Sohn *et al.*, 1991) in France, Japan, Korea and Norway. Thereafter, the disease was documented in a variety of hatchery-reared or cultured warmwater and coldwater marine fish species worldwide (Munday and Nakai, 1997; Munday *et al.*, 2002). In addition, a mass mortality in larval and juvenile European sea bass in French Martinique (Bellance and Gallet de Saint-Aurin, 1988) was probably the first case of the disease.

Almost two decades have passed since the first appearance of VNN in aquaculture. One of the most epoch-making events on research of VNN and the causative agent was the establishment of a cell line (SSN-1) to propagate betanodaviruses (Frerichs *et al.*, 1996), and then a number of fish cell lines were established for the virus. These cell lines made it possible to analyse infectivity of the virus quantitatively, which accelerated all research activities on betanodaviruses and VNN, i.e. the phenotypic and genotypic characteristics, infectivity and host specificity of the virus, infection and transmission mechanisms of the disease and diagnosis and control measures of the disease.

The disease agent

The causative agent of VNN was first purified from diseased larval striped jack, characterized and then identified as a new member of the family *Nodaviridae*, with the name of striped jack nervous necrosis virus (SJNNV) (Mori *et al.*, 1992). Other agents with the same characteristics as SJNNV

were also purified from diseased larval Asian sea bass and European sea bass (Comps *et al.*, 1994) or diseased larvae of a grouper *Epinephelus* sp. (Chi *et al.*, 2001). These nodaviruses from fish were differentiated phylogenetically from nodaviruses isolated from insects (Nishizawa *et al.*, 1995), and the fish viruses were classified as the genus *Betanodavirus* within the family *Nodaviridae* in the 7th *International Committee on Taxonomy of Viruses* (ICTV) report (Ball *et al.*, 2000). SJNNV is the type species of the genus *Betanodavirus*.

Betanodaviruses are non-enveloped and spherical in shape (*c.*25–30 nm in diameter) (Fig. 5.17). The genome consists of two molecules of positive sense ssRNA: RNA1 (3.1 kb) encodes the replicase (110 kDa) and RNA2 (1.4 kb) encodes the coat protein (42 kDa). Both molecules lack poly (A) tails at their 3′-ends (Mori *et al.*, 1992; Comps *et al.*, 1994; Chi *et al.*, 2001; Schneemann *et al.*, 2005). A subgenomic RNA3 (0.4 kb) is derived from RNA1 in infected cells (Sommerset and Nerland, 2004) and encodes a protein with a potent RNA silencing-suppression activity (Iwamoto *et al.*, 2005) (Fig. 5.18). Based on the similarity of the partial RNA2 sequences (*c.*380 bases), betanodaviruses are classified into four major genotypes: designated striped jack nervous necrosis virus (SJNNV)-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and red-spotted grouper nervous necrosis virus (RGNNV)-type (Nishizawa et *al.*, 1995, 1997). A fifth genotype was suggested for a betanodavirus (turbot nodavirus: TNV) isolated from turbot (Johansen *et al.*, 2004). Additional species or strains have been listed tentatively in the genus *Betanodavirus* (Schneemann *et al.*, 2005). Most of them are grouped in either BFNNV genotype or RGNNV genotype. Complete nucleotide sequences of RNA1 and/or RNA2 were reported for SJNNV (Iwamoto *et al.*, 2001), RGNNV (Tan *et al.*, 2001; Iwamoto *et al.*, 2004), BFNNV (Sommerset and Nerland, 2004; Okinaka and Nakai, 2008) and TPNNV (Okinaka and Nakai, 2008).

The genetic differences are related closely to the serotypes by neutralization with polyclonal antibodies, i.e. serotype A for SJNNV genotype, serotype B for TPNNV genotype and serotype C for both BFNNV and RGNNV genotypes (Mori *et al.*, 2003). The host fish is also nearly related to these genotypes (Table 5.4). The RGNNV has been isolated from diseased warmwater fish, while BFNNV has been isolated from diseased coldwater fish (Nishizawa *et al.*, 1997; Aspehaug *et al.*, 1999; Iwamoto *et al.*, 1999; Thiéry *et al.*, 1999; Skliris *et al.*, 2001; Johnson *et al.*, 2002; Chi *et al.*, 2003), suggesting

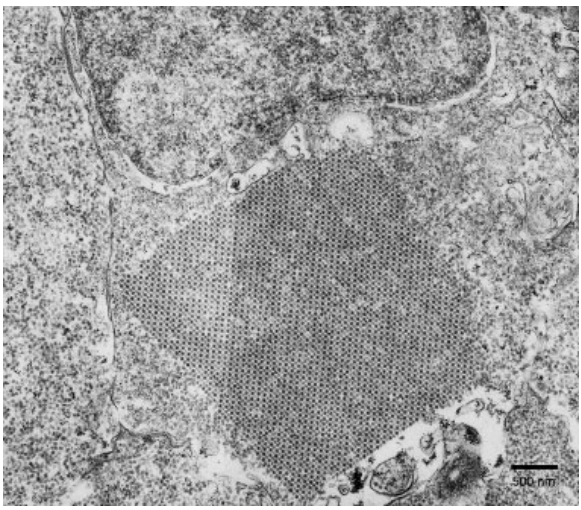


Fig. 5.17. SJNNV in the retinal nerve cell of striped jack larva.

that water temperature is an important factor influencing disease outbreaks. This is partly supported by *in vitro* virus proliferation studies showing the optimum growth temperature of the virus in cultured cells (Iwamoto *et al.*, 2000; Lai *et al.*, 2001; Ciulli *et al.*, 2006; Hata *et al.*, 2007) (Table 5.4).

Geographical distribution and host range

VNN has been reported from all continents, with the exception of South America (Munday *et al.*, 2002). These include Asia (Japan, Korea, Taiwan, China, Philippines, Thailand, Vietnam, Malaysia, Singapore, Indonesia, Brunei, India), Oceania (Australia, Tahiti), the Mediterranean (Bosnia, Croatia, France, Greece, Israel, Italy, Malta, Spain, Portugal, Tunisia), the UK, Scandinavia (Norway) and North America (the USA, Canada).

The number of fish species affected by VNN is increasing steadily. Nineteen fish species (ten families, three orders) including Japanese parrotfish, red-spotted grouper, striped jack, barramundi (Asian sea bass), turbot and European sea bass are described

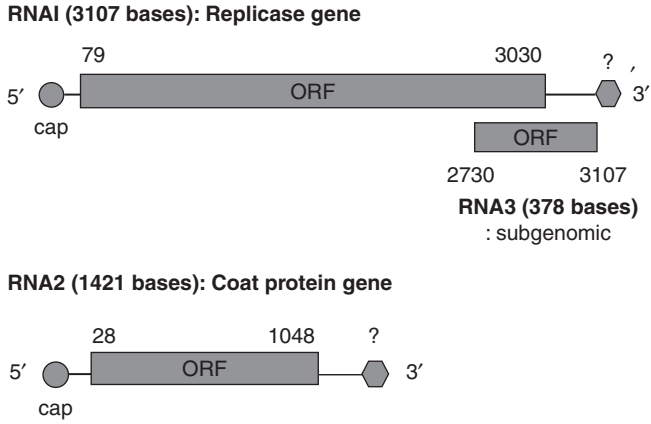


Fig. 5.18. Genome organization of striped jack nervous necrosis virus (SJNNV: type species of the Betanodavirus) (Iwamoto *et al.*, 2001).

Table 5.4. Genotypic and phenotypic variations of betanodaviruses.

Genotype[a]	Serotype[b]	Major host fish	*In vitro* optimum growth temperature[c]
RGNNV (redspotted grouper nervous necrosis virus)	C	Red-spotted grouper and other groupers Asian sea bass European sea bass	25–30°C
SJNNV (striped jack nervous necrosis virus)	A	Striped jack	20–25°C
TPNNV (tiger puffer nervous necrosis virus)	B	Tiger puffer	20°C
BFNNV (barfin flounder nervous necrosis virus)	C	Barfin flounder Japanese flounder Atlantic halibut Atlantic cod Turbot	15–20°C

[a]Nishizawa *et al.* (1995); [b]Mori *et al.* (2003); [c]Iwamoto *et al.* (2000).

as hosts of VNN in an early brief review on the disease (Munday and Nakai, 1997), and 32 species in 16 families (five orders) are listed in the next full-scale review paper (Munday *et al.*, 2002). According to published and unpublished information, these figures have been changed to 44 species, 24 families and eight orders (Table 5.2). Dalla Valle *et al.* (2000) reported the presence of betanodaviruses in cultured gilthead sea bream (*Sparus aurata*), the most important cultured Mediterranean species. However, this species appeared to be relatively resistant to the disease and overt infection of VNN (VER) was not proven (Castric *et al.*, 2001; Ucko *et al.*, 2004). Similarly, no overt VNN has been observed in larval and juvenile rearing and grow-out culture of red sea bream (*Pagrus major*), one of the most popular marine aquaculture species in Japan. This suggests that species in the family *Sparidae* are refractory to the disease, as mentioned by Castric *et al.* (2001). In addition, no overt infection with betanodaviruses has been recorded in salmonids, but one paper suggests that a nodavirus-like agent is associated with cardiomyopathy syndrome of Atlantic salmon (*Salmo salar*) (Grotmol *et al.*, 1997). In addition, Atlantic salmon are susceptible to experimental infection with a betanodavirus (BFNNV genotype) (Korsnes *et al.*, 2005). Some instances of natural infection in freshwater fish species or freshwater farms have also been documented; European eel (*Anguilla anguilla*) and Chinese catfish (*Parasilurus asotus*) (Chi *et al.*, 2003), European sea bass (Athanassopoulou *et al.*, 2003) and sturgeon (*Acipenser gueldestaedti*) (Athanassopoulou *et al.*, 2004) and tilapia (*O. niloticus*) (Bigarre *et al.*, 2009). Although a nodavirus infection in guppy was reported, the viral aetiology was not fulfilled by experimental infection (Hegde *et al.*, 2003). Experimental infections showed that other salt-water and freshwater fish were susceptible to the virus; spotted wolf-fish (*Anarhichas minor*) (Johansen *et al.*, 2003; Sommer *et al.*, 2004), mangrove red snapper (*Lutjanus argentimaculatus*) and milkfish (*Chanos chanos*) (Maeno *et al.*, 2007), medaka (*Oryzias latipes*) and some other ornamental freshwater fish species (Furusawa *et al.*, 2006, 2007), suggesting new hosts of VNN.

Economic importance of the disease

VNN is one of the most important limiting factors in successful seed production of marine aquaculture fish species, because of its wide range of susceptible host species (Table 5.5) and higher virulence, often causing severe mortalities up to 100%. At the National Research Institute of Aquaculture, Japan (formerly Japan Sea-Farming Association), VNN of striped jack first appeared in the facility in 1989 and no juveniles of striped jack were produced in 1990. Thereafter, heavy larval mortalities continued for several years until the establishment of control methods (Mori *et al.*, 1998; Muroga *et al.*, 1998; Mushiake and Arimoto, 2000). Furthermore, some species of groupers which have a high value in Asian countries are still susceptible to the virus even at marketable stages. Mortality in a grouper species, e.g. seven-band grouper, may reach more than 50% during net-cage culture, leading to serious economic losses (Fukuda *et al.*, 1996). VNN (VER) is also a major threat in European sea bass and Atlantic halibut (*Hippoglossus hippoglossus*) aquacultures in the Mediterranean and Norway, respectively (Agius and Tanti, 1997; Bergh *et al.*, 2001).

Diagnostic methods

VNN occurs at early developmental stages (larvae and juveniles) in susceptible fish species. There are considerable variations in the age at which disease is first noted and the period over which mortality occurs (Munday *et al.*, 2002; OIE, 2006). In general, the earlier the signs of disease occur, the greater is the rate of mortality. In striped jack, the earliest outbreak of the disease is at 1 d.p.h. and the latest occurrence of new outbreaks is at 20 d.p.h. (8 mm in total length), resulting in almost complete loss of

Table 5.5. Fish species affected by viral nervous necrosis (VNN).

Family	Host fish species	Reference
Acipenseridae	Sturgeon (*Acipenser gueldestaedti*)	Athanassopoulou *et al*. (2004)
Anguillidae	European eel (*Anguilla anguilla*)	Chi *et al*. (2003)
Chanidae	Milkfish (*Chanos chanos*)	Maeno *et al*. (2007)
Siluridae	Chinese catfish (*Parasilurus asotus*)	Chi *et al*. (2003)
Gadidae	Atlantic cod (*Gadus morhua*)	Starkey *et al*. (2001); Johnson *et al*. (2002); Patel *et al*. (2007)
	Pacific cod (*Gadus macrocephalus*)	Unpublished
	Haddock (*Melanogrammus aeglefinus*)	Gagné *et al*. (2004)
Platycephalidae	Bartail flathead (*Platycephalus indicus*)	Song *et al*. (1997)
Centropomatidae	Asian sea bass (*Lates calcarifer*)	Glazebrook *et al*. (1990); Renault *et al*. (1991); Munday *et al*. (1992); Zafran *et al*. (1998); Azad *et al*. (2005)
	Japanese sea bass (*Lateolabrax japonicus*)	Jung *et al*. (1996)
Percichthydae	European sea bass (*Dicentrarchus labrax*)	Breuil *et al*. (1991); Le Breton *et al*. (1997); Athanassopoulou *et al*. (2003)
Serranidae	Red-spotted grouper (*Epinephelus akaara*)	Mori *et al*. (1991); Chi *et al*. (1997)
	Yellow grouper (*E. awoara*)	Lai *et al*. (2001)
	Seven-band grouper (*E. septemfasciatus*)	Sohn *et al*. (1991); Fukuda *et al*. (1996)
	Black-spotted grouper (*E. fuscogutatus*)	Chi *et al*. (1997)
	Dusky grouper (*E. marginatus*)	Munday *et al*. (2002)
	Brown-spotted grouper (*E. malabaricus*)	Danayadol and Direkbusarakom (1995); Boonyaratpalin *et al*. (1996); Lin *et al*. (2001)
	Kelp grouper (*E. moara*)	Nakai *et al*. (1994)
	Greasy grouper (*E. tauvina*)	Chua *et al*. (1995); Chew-Lim *et al*. (1998)
	Dragon grouper (*E. lanceolatus*)	Lin *et al*. (2001)
	Orange-spotted grouper (*E. coioides*)	Maeno *et al*. (2002)
	White grouper (*E. aeneus*)	Ucko *et al*. (2004)
	Humpback grouper (*Chromileptes altivelis*)	Zafran *et al*. (2000)
Malacanthidae	Japanese tilefish (*Branchiostegus japonicus*)	Unpublished
Latridae	Striped trumpeter (*Latris lineata*)	Munday *et al*. (2002)
Carangidae	Striped jack (*Pseudocaranx dentex*)	Mori *et al*. (1992)
	Yellow-wax pompano (*Trachinotus falcatus*)	Chi *et al*. (2001)
Lutjanidae	Mangrove red snapper (*Lutjanus argentimaculatus*)	Maeno *et al*. (2007)
Sciaenidae	Red drum (*Sciaenops ocellatus*)	Ucko *et al*. (2004)
	Shi drum (*Umbrina cirrosa*)	Bovo *et al*. (1999)
	White sea bass (*Atractoscion nobilis*)	Curtis *et al*. (2001)
Oplegnathidae	Japanese parrotfish (*Oplegnathus fasciatus*)	Yoshikoshi and Inoue (1990)
	Rock porgy (*O. punctatus*)	Muroga *et al*. (1998)
Mugilidae	Grey mullet (*Mugil cephalus*)	Ucko *et al*. (2004)
Cichlidae	Tilapia (*Oreochromis niloticus*)	Bigarre *et al*. (2009)
Eleotridae	Sleepy cod (*Oxyeleotris lineolatus*)	Munday *et al*. (2002)
Rachycentridae	Cobia (*Rachycentron canadum*)	Chi *et al*. (2003)
Scombridae	Bluefin tuna (*Thunnus thynnus*)	Unpublished

Table 5.5.

Family	Host fish species	Reference
Pleuronectidae	Barfin flounder (*Verasper moseri*)	Watanabe *et al.* (1999)
	Atlantic halibut (*Hippoglossus hippoglossus*)	Grotmol *et al.* (1995, 1997); Starkey *et al.* (2000)
Bothidae	Japanese flounder (*Paralichthys olivaceus*)	Nguyen *et al.* (1994)
	Turbot (*Pesta maxima*)	Bloch *et al.* (1991); Johansen *et al.* (2004)
Soleidae	Dover sole (*Solea solea*)	Starkey *et al.* (2001)
Tetraodontidae	Tiger puffer (*Takifugu rubripes*)	Nakai *et al.* (1994)

Modified from Munday *et al.* (2002).

larvae (Mori *et al.*, 1992; Arimoto *et al.*, 1994). The susceptible period for VNN of a grouper, *E. coioides*, is the larval and post-larval stage (14–40 d.p.h.) (Lin *et al.*, 2007). However, mortality is usually not seen until about 30 d.p.h. in European sea bass and new disease outbreaks occur even in marketable size (Le Breton *et al.*, 1997). Seven-band grouper suffer the disease throughout all developmental stages, from larvae to marketable size (up to 1.8 kg in body weight) (Fukuda *et al.*, 1996; Tsuchihashi *et al.*, 2002). Significant mortalities at grow-out stages also occur in European sea bass (Le Breton *et al.*, 1997; Bovo *et al.*, 1999), Atlantic halibut (Aspehaug *et al.*, 1999) and Atlantic cod (*Gadus morhua*) (Patel *et al.*, 2007). Mortality of juvenile or older fish usually does not reach 100%, indicating the age dependence of susceptibility.

There are no gross clinical signs on the body surface and gills of affected fish at any stages. The affected juveniles or older fish show a variety of erratic swimming behaviours such as spiral, whirling, belly-up floating with inflation of swim bladder (Figs 5.19 and 5.20) or lying down at rest, but usually it is not easy to find such swimming abnormalities in affected larvae. Histopathologically, the disease is characterized by severely extended necrosis and vacuolation of the central nervous system (brain, spinal cord) and retina (Fig. 5.21), but sometimes fish at the early larval stage lack conspicuous vacuolation in the tissues (Munday *et al.*, 2002).

Presumptive diagnosis of VNN is based on the appearance of vacuoles in the brain, spinal cord and/or retina as seen by light microscopy. However, individual fish showing only a few vacuoles in the nervous tissues pose a difficulty in diagnosis. In electron microscopy, the virus is associated mainly with vacuolated cells, arranged intracytoplasmically in paracrystalline arrays or as aggregates. The virions, about 25 nm ranging from 20 to 34 nm, might be membrane-bound by endoplasmic reticulum in the cytoplasm of cells, which were identified as neurons, astrocytes, oligodendrocytes and microglia (Yoshikoshi and Inoue, 1990; Bloch *et al.*, 1991; Grotmol *et al.*, 1997). Confirmatory diagnosis is made by immunostaining methods, fluorescent antibody test (FAT) or immunohistochemistry (IHC), using polyclonal or monoclonal antibodies (Nguyen *et al.*, 1996; Grotmol *et al.*, 1999; Nishizawa *et al.*, 1999; Tanaka *et al.*, 2004; Shieh and Chi, 2005; OIE, 2006). There have been some reports on enzyme-linked immunosorbent assay (ELISA) to detect viral antigens in diseased fish: SJNNV in striped jack larvae (Arimoto *et al.*, 1992), RGNNV in grouper *E. coioides* (Shieh and Chi, 2005) and RGNNV in Asian sea bass (Fenner *et al.*, 2006). The presence of SJNNV antigens in gonads of apparently healthy broodstocks was also demonstrated by ELISA (Arimoto *et al.*, 1992, Mushiake *et al.*, 1992).

Reverse-transcription polymerase chain reaction (RT-PCR) is the most rapid and convenient method to diagnose clinically affected fish, and nested PCR is also useful to diagnose, particularly, asymptomatic fish (broodstock). There are a number of reported



Fig. 5.19. Belly-up floating seven-band grouper affected with VNN.



Fig. 5.20. Swim-bladder inflation of seven-band grouper.

RT-PCR and nested PCR methods with different primers and protocols, but the methods are not standardized (Nishizawa *et al*., 1994; Thiéry *et al*., 1999; Grotmol *et al*., 2000; Huang *et al*., 2001; Gomez *et al*., 2004; Cutrin *et al*., 2007). Among them, the primer set (R3 and F2; Nishizawa *et al*., 1994) designed to amplify the variable region (*c*.380 bases) of SJNNV coat protein gene is useful for detection of almost all genotypic variants, with only one exception (Thiéry *et al*., 1999). However, these PCRs have to be followed by the other methods, either histopathology with immunostaining or virus isolation in cell culture, for a confirmative diagnosis.

Several fish cell lines are now available to isolate and propagate betanodaviruses; SSN-1 (Frerichs *et al*., 1996) and E-11 (Iwamoto *et al*., 2000) derived from striped snakehead (*Ophicephaus striatus*), GF-1 (Chi *et al*., 1999) and other cell lines derived from groupers (Lai *et al*., 2001, 2003; Qin *et al*., 2006), TV-1 and TF derived from turbot (Aranguren *et al*., 2002) and SAF-1 derived from gilthead sea bream (Bandín *et al*., 2006). Virus identification in cell culture showing CPEs is performed either by serological assays including a virus-neutralizing test (Mori *et al*., 2003) or by molecular assays.

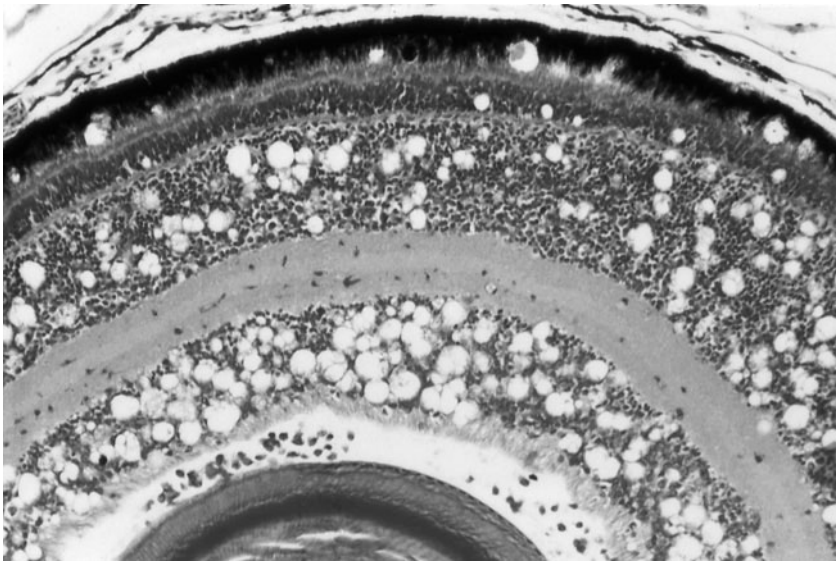


Fig. 5.21. Extensive vacuolation in the retina of red-spotted grouper.

Serodiagnosis may be useful to know the infection status of the fish, though difficulties exist in practice. ELISA methods to detect anti-betanodavirus antibodies in fish were reported. Mushiake *et al.* (1992) used an indirect ELISA to detect anti-SJNNV antibodies in plasma of striped jack broodstock. Detection of antibodies for broodstock screening was also reported in European sea bass (Breuil and Romestand, 1999; Breuil *et al.*, 2000) and barfin flounder (*Verasper moseri*) (Watanabe *et al.*, 2000).

Pathogenesis and immunity

A close relationship is recognized between the genotypes and host specificity in betanodaviruses; e.g. SJNNV is pathogenic to striped jack larvae but not to seven-band grouper larvae, while RGNNV is pathogenic to seven-band grouper larvae but not to striped jack larvae. An infectious RNA transcription system was constructed for SJNNV and RGNNV, and infection experiments using reassortant viruses comprising RNA1 (RNA-dependent RNA polymerase gene) and RNA2 (coat protein gene) revealed that the host specificity of SJNNV and RGNNV was controlled by the RNA2 (Iwamoto *et al.*, 2001, 2004). Furthermore, infection experiments using RNA2 chimeric viruses from SJNNV and RGNNV indicated that the variable region of RNA2 determined host specificity in these viruses (Ito *et al.*, 2008).

In larval production of striped jack, no difference was noticed in mortality caused by SJNNV (*in vitro* optimum growth temperature: 20–25°C) infection under rearing water temperatures ranging from 20 to 26°C. Infection experiments with RGNNV (*in vitro* optimum: 25–30°C) indicated that rearing water temperature (16–28°C) influenced development of the disease in red-spotted grouper. Higher mortality and earlier appearance of the disease in seven-band grouper were observed at higher temperatures (Tanaka *et al.*, 1998), but water temperature higher than 31°C inhibited the proliferation of RGNNV in humpback grouper (*Cromileptes altivelis*) (Yuasa *et al.*, 2007a). On the other hand, the infection of Atlantic halibut by Atlantic halibut nodavirus (BFNNV genotype, *in vitro* optimum: 15–20°C) occurred at 6°C (Grotmol *et al.*, 1999).

Nguyen *et al.* (1996) indicated that the initial multiplication site of SJNNV in

striped jack larvae was the spinal cord, particularly the area just above the swim bladder, from which the virus spread to the brain and finally to the retina; but the role of skin as a portal of entry for SJNNV remains unclear. Grotmol *et al.* (1999) suggested that the portal of entry of BFNNV into Atlantic halibut larvae was the intestinal epithelium and the route of infection to the central nervous system was axonal transport to the brain through cranial nerves. On the other hand, Tanaka *et al.* (2004) indicated that seven-band grouper at grow-out stages could be infected with RGNNV via the pernasal route.

Castric *et al.* (2001) demonstrated that juvenile sea bream were refractory to experimental infection with RGNNV, but the virus was isolated from asymptomatic fish. Persistent and subclinical nodavirus infection was also reported in juvenile Atlantic halibut and spotted wolf-fish infected with BFNNV (Johansen *et al.*, 2002, 2003). A cell line (BB) derived from the brain tissue of Asian sea bass that had survived VNN was reported to be persistently infected with RGNNV, suggesting association of interferon to the persistence of the virus (Chi *et al.*, 2005). PCR-based methods demonstrated the presence of betanodaviruses in apparently healthy wild or farmed marine fish (Gomez *et al.*, 2004; Baeck *et al.*, 2007; Cutrin *et al.*, 2007). These subclinically infected fish are important as a potential carrier of the virus.

Control, treatment and epizootiology

The virus is highly resistant to various environmental conditions and can survive for a long time in seawater (Arimoto *et al.*, 1996; Frerichs *et al.*, 2000). The disease is reproduced easily in healthy fish by cohabitation with infected fish or immersion in virus suspension, as well as injection methods (Nguyen *et al.*, 1996; Grotmol *et al.*, 1999; Peducasse *et al.*, 1999). Therefore, basically, the transmission mode of infection is horizontal through influent and rearing water, including the possibilities of virus transmission via utensils, vehicles and other human activities. In order to prevent such horizontal transmission, some disinfectants are useful for inactivating the virus: acid peroxygen, benzalkonium chloride, iodine, ozone and sodium hypochlorite (Arimoto *et al.*, 1996; Frerichs *et al.*, 2000).

There is also evidence of vertical transmission of infection from broodstock to offspring in striped jack, European sea bass, Asian sea bass, barfin flounder, Atlantic halibut and seven-band grouper (Arimoto *et al.*, 1992; Comps *et al.*, 1994; Mushiake *et al.*, 1994; Watanabe *et al.*, 2000). Washing of fertilized eggs in ozone-treated seawater and disinfection of rearing water by ozone are effective for controlling the disease in larvae production of striped jack, barfin flounder, Atlantic halibut and seven-band grouper (Mori *et al.*, 1998; Watanabe *et al.*, 1998; Grotmol and Totland, 2000; Tsuchihashi *et al.*, 2002). It is also important to reduce stress factors by improvement of spawning induction methods including food for broodstock (Mushiake *et al.*, 1994). The Japan Sea-Farming Association started to investigate VNN in larval striped jack in 1990 and succeeded in controlling the disease by elimination of virus-carrying broodstocks and disinfection of fertilized eggs and rearing water (Mori *et al.*, 1998; Mushiake and Arimoto, 2000). The established procedures proved to be effective for VNN in larval seven-band grouper (Tsuchihashi *et al.*, 2002).

However, some fish species such as groupers and sea bass are highly susceptible to the virus (RGNNV) at the grow-out stage, and VNN causes severe mortality during net-pen culture in the open sea. This infection may be caused by the virus shed from subclinically or persistently infected cultured or wild fish (Castric *et al.*, 2001; Barker *et al.*, 2002; Johansen *et al.*, 2003; Gomez *et al.*, 2004; Chi *et al.*, 2005; Cutrin *et al.*, 2007; Sakamoto *et al.*, 2008). Therefore, an efficacious vaccination system is essential to prevent the disease during the grow-out stages in net-pen culture. Some studies demonstrated that injection immunization with a recombinant viral coat protein expressed in *Escherichia coli* (Húsgard *et al.*, 2001; Tanaka *et al.*, 2001; Sommerset

et al., 2005), synthetic peptide (Coeurdacier *et al.*, 2003), virus-like particles (VLPs) expressed in a baculovirus expression system (Liu *et al.*, 2006; Thiéry *et al.*, 2006) or inactivated virus (Yamashita *et al.*, 2005, 2009a) was effective in controlling the disease. A correlation between neutralizing antibody titres and protection against virus infection was reported. Thiéry *et al.* (2006) revealed that European sea bass immunized with the VLPs and having neutralizing antibody titres ranging from 1:40 to 1:1280 were protected against RGNNV challenge, with RPS greater than 60%. Yamashita *et al.* (2009b) deduced in the immunization experiment of seven-band grouper with the inactivated virus that the minimum neutralizing antibody titre giving significant protection against RGNNV infection was 1:200. In contrast, immunization of juvenile turbot (2.2 g) with the recombinant Atlantic halibut nodavirus (AHNV; BFNNV genotype) coat protein and non-oil adjuvant induced protection against AHNV infection, despite the lack of antibodies (Sommerset *et al.*, 2005). Recently, the efficacy of oral immunization with RGNNV recombinant coat protein-containing *Artemia* nauplii (Lin *et al.*, 2007) or bath immunization with RGNNV-inactivated vaccine (Kai and Chi, 2008) was demonstrated in larval orange-spotted grouper, *E. coioides*. All these types of vaccine are plausible candidates for control of the disease. Particularly, oral and bath administrations are useful for larval/juvenile VNN. However, whatever vaccine is selected, the construction of a practical vaccination system will require more detailed data on various parameters such as administration route, effective dose and duration of vaccine. In addition, it was also documented in experiments using seven-band grouper that a primary infection with an avirulent aquabirnavirus (*Birnaviridae*) as interferon inducer effectively suppressed a secondary betanodavirus infection (Pakingking *et al.*, 2005), and furthermore that simultaneous injection with the aquabirnavirus and RGNNV-inactivated vaccine conferred rapid onset of non-specific protection and long-lasting specific protection against the disease (Yamashita *et al.*, 2009a).

Conclusions and recommendations for future studies

More than 200 papers on VNN infections and related subjects have been published in the past two decades. Current knowledge on the disease and causative betanodaviruses accumulated through these studies has led to the development of many useful diagnostic and control methods. However, much remains to be done in order to reveal the molecular characteristics of the virus, interaction between host fish and the virus, and the transmission mechanisms in natural environments, and to establish more practical control procedures for the disease, especially in reducing severe economic damage during net-pen culture in the open sea. A commercially available vaccine for VNN is essential for future development within marine aquaculture.

Iridovirus Diseases

Introduction

The family *Iridoviridae* is currently subdivided into 5 genera according to the ICTV (Chinchar *et al.*, 2005), although its taxonomy is still not fully resolved. Viruses in the genus *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus* of the family are known as the causative agent of fish iridovirus disease. Iridoviruses have an icosahedral virion with an internal lipid membrane ranging from about 120 to 200 nm in diameter, containing a single, linear, double-stranded DNA molecule of 140–303 kbp. Virion formation takes place in the cytoplasm of the infected cell.

The genus *Ranavirus* of which the type species is frog virus 3 (FV3) includes epizootic haematopoietic necrosis virus (EHNV) in Australia (Langdon *et al.*, 1986b; Eaton *et al.*, 1991; Hedrick *et al.*, 1992), which is the causative virus of an OIE listed disease (OIE, 2009a), European catfish virus (ECV) and European sheatfish virus (ESV) in Europe (Ahne *et al.*, 1989; Pozet *et al.*, 1992) and Santee-Cooper ranavirus (largemouth

bass virus [LMBV]) in America (Plumb *et al.*, 1996). Viruses in this group are becoming increasingly important as pathogens of feral, cultured and ornamental teleosts. They are endotheliotropic and can induce severe systemic disease in susceptible fish species, characterized by necrotic lesions in vascular walls and visceral organs.

The *Lymphocystivirus* includes one species of lymphocystis disease virus 1 (LCDV-1) and tentative species of lymphocystis disease virus 2 (LCDV-2) (Chinchar *et al.*, 2005). Infection results in benign, wart-like lesions comprising grossly hypertrophied cells occurring mostly in the skin and fins. The disease has been observed in over 100 teleost species, although virus species other than LCDV-1 or LCDV-2 may cause a similar clinical disease (Chinchar *et al.*, 2005).

The *Megalocytivirus* consists of one species, namely, infectious spleen and kidney necrosis virus (ISKNV) isolated from freshwater fish. It includes red sea bream iridovirus (RSIV) from marine fish inducing red sea bream iridoviral disease (RSIVD), which are OIE listed diseases (OIE, 2009a), and also many viruses given a name after the affected fish, such as African lampeye iridovirus, sea bass iridovirus and Taiwan grouper iridovirus (OIE, 2006). The disease caused by the virus in this group is characterized by the appearance of enlarged cells in spleen, heart, haematopoietic tissue, gills and digestive tract.

This section, iridovirus diseases, describes systemic iridovirus diseases, mostly EHN, caused by ranaviruses, and RSIVD, because they are listed by the OIE (2009a) and are a potential threat to aquaculture.

Systemic iridovirus diseases caused by ranaviruses

The disease agent

The genus *Ranavirus* currently recognizes six species: ambystoma tigrinum virus, Bohle iridovirus, epizootic haematopoietic necrosis virus, European catfish virus, frog virus 3 and Santee-Cooper ranavirus according to the ICTV (Chinchar *et al.*, 2005). The type species is the frog virus 3 including FV-3 and several other amphibian and reptile viruses. European catfish virus and Santee-Cooper ranavirus include European catfish virus (ECV) and European sheatfish virus (ESV), doctor fish virus (DFV), guppy virus 6 (GV6) and Santee-Cooper ranavirus (=largemouth bass virus [LMBV]), respectively. Singapore grouper iridovirus (SGIV; Qin *et al.*, 2003) is placed as one of the tentative species in this genus. As Hedrick *et al.* (1992) suggested that epizootic haematopoietic necrosis virus (EHNV) and ictalurid isolates (ECV, ESV) were strains of FV-3, several piscine, reptilian and amphibian ranavirus isolates show serological and genetic relatedness to FV-3. In addition, the Bohle iridovirus (BIV) induced 100% mortality in bath infected barramundi (*L. calcarifer*) (Moody and Owens, 1994). Jensen *et al.* (2009) demonstrated that EHNV, ESV, pike-perch iridovirus (PPIV; Tapiovaara *et al.*, 1998) and New Zealand eel virus (NZeelV; Bovo *et al.*, 1999) caused significant mortality of pike (*E. lucius*) and FV-3 could infect the fish after bath challenge. Reports indicate the possible importance of amphibian ranaviruses for finfish aquaculture and also aquatic amphibian communities themselves, and consequently amphibian ranavirus has been listed in the OIE Aquatic Animal Health Code since 2008 (OIE, 2009a). Chinchar (2002), Chinchar *et al.*, (2005, 2009) and Whittington *et al.* (2010) describe reviews of ranavirus. EHN and EHNV were reviewed by Wolf (1988) and McAllister (1993) and a review of LMBV was presented by Grizzle and Brunner (2003).

Fish ranaviruses EHNV (Langdon *et al.*, 1986b), ECV (Pozet *et al.*, 1992) and LMBV (Plumb *et al.*, 1996) are distinct agents that can be differentiated using genomic analysis (Mao *et al.*, 1997, 1999; Ahne *et al.*, 1998; Hyatt *et al.*, 2000), although they have similar hexagonal morphology and size (slightly over 130–140 nm), number and weight of structural polypeptides and common antigens (Hedrick *et al.*, 1992). The major capsid protein (MCP) is the predominant structural component of the virus particle, comprising 40–45% of the total protein (Willis *et al.*,

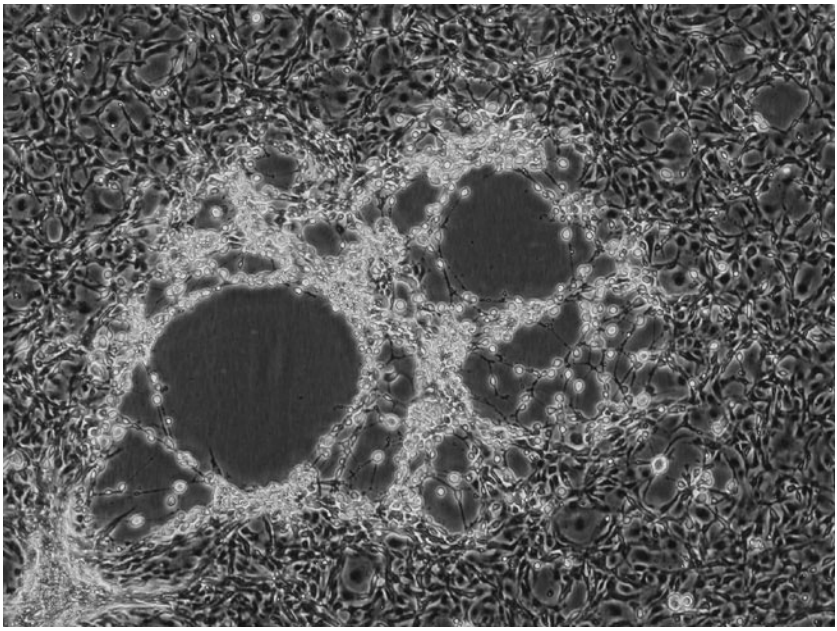


Fig 5.22. CPE on BF-2 cells following infection with EHNV.

1977), but antigenic panel assay using monoclonal antibodies demonstrates that EHNV 50 kDa MCP does not act as an immunodominant antigen in the particle and two peptides associated with the surface of the virion, sized 15 and 18 kDa, are able to elicit a strong immune response in mice (Monini and Ruggeri, 2002). They replicate in cytoplasm and induce inclusion bodies as centres of virus production. The CPE in cell cultures is characterized by focal rounding up of cells from substrate and cell lysis, expansion of these changes and destruction of the cell monolayer.

EHNV replicates in many fish cell lines, including BF-2, EPC, FHM and CHSE-214, at temperatures ranging from 15 to 22°C (Crane *et al.*, 2005) (Fig. 5.22). Langdon and Humphrey (1987) and Langdon *et al.* (1988) obtained the highest virus yields in FHM cells. BF-2 at 22°C is recommended by the OIE (2006) for the isolation of EHNV. ESV is isolated readily in BF-2 and FHM cells at 20–30°C. Iridovirus of ornamental tropical fish replicates in the same cell lines. LMBV propagate in BF-2 and FHM cells (Piaskoski *et al.*, 1999; McClenahan *et al.*, 2005). SGIV grows in grouper embryo, sea bass fry and FHM cell lines (Qin *et al.*, 2003) and other marine fish cell lines reported recently (Lai *et al.*, 2003; Qin *et al.*, 2006; Huang *et al.*, 2009).

Geographical distribution and host range

Members of the ranavirus from freshwater fish were isolated on three continents. EHNV affects redfin perch (*Perca fluviatilis*) and rainbow trout in Australia (Victoria, New South Wales and South Australia) (Langdon *et al.*, 1986a,b, 1988; Langdon and Humphrey, 1987; Whittington *et al.*, 1996), which is geographically distinct from BIV being present only in Queensland (Speare and Smith, 1992). ESV was isolated in Germany (Ahne *et al.*, 1989). ECV caused mortality of *A. melas* in France (Pozet *et al.*, 1992) and in Italy (Bovo *et al.*, 1993). In Santee-Cooper ranavirus, GV6 and DFV were isolated from imported guppy and doctor fish (*Labroides dimidatus*) in California, USA, but those from exotic ornamental fish were suspected

to be part of a putative transcontinental transmission (Hedrick and McDowell, 1995). LMBV was first isolated from largemouth bass in Lake Weir, Florida, USA, in 1991 (Grizzle *et al.*, 2002) and from a die-off of adult largemouth bass in a reservoir in South Carolina (Plumb *et al.*, 1996) and subsequently was detected mostly from wild fish associated with die-offs in eastern states (Plumb *et al.*, 1999; Hanson *et al.*, 2001; Grizzle and Brunner, 2003; Groocock *et al.*, 2008; Southard *et al.*, 2009). Largemouth bass and striped bass are susceptible to LMBV in experimental infection (Plumb and Zilberg, 1999). The ranavirus from marine fish were isolated in Asia. SGIV was isolated from diseased grouper, *E. tauvina*, in Singapore (Qin *et al.*, 2003) and a similar virus named grouper iridovirus (GIV) was isolated from diseased *E. awoara* in Taiwan (Murali *et al.*, 2002; Lai *et al.*, 2003).

Natural EHNV infections are known from only two fish species, redfin perch and rainbow trout (Langdon *et al.*, 1986a,b, 1988; Langdon and Humphrey, 1987); however, a number of other finfish species are susceptible to EHNV in experimental bath inoculation: Macquarie perch (*Macquaria australasica*), silver perch (*B. bidyanus*), mosquito fish (*Gambusia affinis*), mountain galaxias (*Galaxias olidus*) and pike (*E. lucius*) (Langdon, 1989; Jensen *et al.*, 2009). Interestingly, Ariel and Jensen (2009) demonstrated that European stocks of redfin perch and rainbow trout did not show significant mortality by bath challenge with EHNV. Some species, for example goldfish (*C. auratus*) and common carp, are resistant.

Economic importance of the disease

The economic damage due to EHN and ictalurid ranavirus is deemed rare, but they can cause 100% mortality in young susceptible warmwater hosts.

Largemouth bass is a fish species for angling and there are no reports of damage to the wild fish population due to LMBV. But it has caused fish die-off in several eastern USA states and there has been a decline in the number of largemouth bass larger than 2.3 kg in some infected populations (Grizzle and Brunner, 2003).

Grouper are one of the important species being aquacultured in East and Southeast Asian countries and are a high-priced and popular seafood fish. SGIV can cause significant losses of up to 50–90% of grouper sized from fry to marketable fish (Chua *et al.*, 1994; Qin *et al.*, 2003).

Diagnostic methods

Specific diagnosis of EHN is based generally on the isolation of EHNV in cell culture, followed by its immunological or nucleic acid-based identification such as PCR (OIE, 2006). This approach for EHNV is applicable to other viruses in the ranavirus group, being combined with a specific PCR. Presumptive diagnosis is based on clinical signs and virus isolation in BF-2 or other susceptible cells. Isolation and propagation of ranaviruses in cell culture is reported in detail by Ariel *et al.* (2009). Sample homogenate of liver, anterior kidney and spleen is inoculated to BF-2 cells and incubated at 22°C for 6 days. Cultures without CPE are passed at least once by inoculating supernatant of the culture after being frozen overnight at –20°C and thawed on to fresh cells. Identification of virus in cell culture in the OIE manual (2006) is based on ELISA tests (Whittington and Steiner, 1993) or PCR-restriction endonuclease analysis (REA) (Marsh *et al.*, 2002). It should be noted that antibodies used in immunological tests cross-react with all known ranaviruses (Hedrick *et al.*, 1992; Ahne *et al.*, 1993; Hengstberger *et al.*, 1993; Hedrick and McDowell, 1995; Hyatt *et al.*, 2000). Gould *et al.* (1995) reported a PCR for the detection of EHNV and BIV, and a PCR-REA developed by Marsh *et al.* (2002) differentiated EHNV from ECV, ESV, FV-3, BIV, Wamera virus (WV) and Gutapo virus (GV) with two primer sets designed in the major capsid protein (MCP) gene. The amplicons are digested with three kinds of endonuclease, and an electorophoresis profile can be interpreted for the identification. Alternatively, a PCR-targeting MCP is used for amplification of the target region of 580 bp and consequent sequencing of the

amplicon can be used for specific detection of EHNV, ESV, ECV, GV6, DFV and a range of amphibian ranaviruses (Hyatt *et al.*, 2000). Another PCR-REA method based on MCP and neurofilament triplet H1-like protein gene sequences of 11 strains of piscine and amphibian ranaviruses was developed by Holopainen *et al.* (2009). A real-time PCR assay for the detection and differentiation of Australian and European ranaviruses has been developed (Pallister *et al.*, 2007). PCR detection methods (Grizzle *et al.*, 2003; McClenahan *et al.*, 2005) and quantitative PCR assays (Goldberg *et al.*, 2003; Getchell *et al.*, 2007) have been reported for LMBV. A loop-mediated isothermal amplification (LAMP) method for detecting SGIV has been reported (Mao *et al.*, 2008).

Genome structure and transcription

Ranavirus has a single, linear dsDNA molecule of 150–170 kbp. The genome is permuted circularly and approximately 30% terminally redundant. The complete genome sequences of six ranaviruses have been reported: ambystoma tigrinum virus (ATV): 106,332 bp (AY150217; Jancovich *et al.*, 2003); FV3: 105,903 bp (AY548484; Tan *et al.*, 2004); tiger frog virus (TFV): 105,057 bp (AF389451; He *et al.*, 2002a); soft-shelled turtle iridovirus (STIV): 105,890 bp (EU627010; Huang *et al.*, 2009); SGIV: 140,131 bp (AY521625; Song *et al.*, 2004); GIV: 139,793 bp (AY666015; Tsai *et al.*, 2005). Namely, the unit genome size for amphibian virus is approximately 105 kbp with a G+C content of around 55%, and for grouper virus it is approximately 140 kbp with 49% G+C content. Cytosines within the dinucleotide sequence CpG are methylated by a virus-encoded cytosine DNA methyltransferase of the amphibian viruses, which has not been found in grouper viruses.

Approximately 30 structural proteins have been detected in FV3 virions, whereas sequence analysis of amphibian virus (ATV, FV3, TFV and STIV) predicted slightly more than 100 ORFs, respectively. In the virion of grouper virus, SGIV, comprehensive and precise identification showed 44 viral proteins (Song *et al.*, 2006), although predicted ORFs in grouper viruses were 162 for SGIV and 120 for GIV. Extensive annotation study on iridoviruses including five ranaviruses has been carried out (Eaton *et al.*, 2007), but further studies are needed to understand iridoviral gene function fully.

Transcription of iridoviral DNA is a coordinated sequential process involving the production and regulation of mRNAs that can be classified into immediate-early, delayed-early and late genes according to their temporal synthesis on infection (see Williams, 1996; Chinchar, 2002; Chinchar *et al.*, 2005, 2009; Williams *et al.*, 2005; Chen *et al.*, 2006; Teng *et al.*, 2008; Majji *et al.*, 2009).

Pathogenesis and immunity

Fish ranaviruses are endotheliotropic and cause haemorrhagic diathesis, oedema and peripheral circulatory failure. The immunological response in fish and rabbits generally does not include neutralizing antibodies.

EHN appeared during the spring of 1984 in a lake and caused severe mortality among juvenile redfin perch. The disease is less serious in farmed rainbow trout. Experimentally infected 35- to 45-day-old redfin perch develop depression, skin darkening and erratic swimming and die after 4–5 days (Langdon, 1989). Some have erythema around the brain and nostrils. Clinical signs in disease outbreaks described by Langdon and Humphrey (1987) were identical, except for skin ulcers invaded by fungus in some fish. Focal necrosis is a consistent finding in haematopoietic kidney and liver of naturally and experimentally infected fish. In the spleen and pancreas, this sign is variable. Necrotic haematopoietic cells are disseminated in all vessels. Lesions in other susceptible species are inconstant (Langdon, 1989). The most consistent is the necrosis in haematopoietic kidney and liver. A description of gross lesions in redfin perch by Reddacliff and Whittington (1996) includes haemorrhage around the bases of fins, focal haemorrhage in gills, oedema and multiple necrotic foci in liver. Microscopic changes consist of focal to extensive necrosis in haematopoietic kidney, liver, spleen, heart,

pancreas and lamina propria of the intestine. Thrombosis, haemorrhage and fibrinous exudate are common in gills. Lesions in rainbow trout are similar but milder. Redfin perch survivors from natural disease outbreaks are resistant to challenge with EHNV (Langdon, 1989). The epizootiology of EHN in rainbow trout suggests that this species is incapable of developing long-lasting resistance (OIE, 2006).

The sheatfish iridovirus disease (iridovirous wels disease [IWD]) caused by ESV is characterized by loss of appetite, apathy, ataxia (including rapid spiral swimming), petechial haemorrhage in skin and internal organs and generalized destruction of haematopoietic tissues in the kidney and spleen. The cumulative fry mortality in a recirculation system was 100% (Ahne *et al.*, 1989). Fry infected by bath in virus suspension and by cohabitation also succumbed to high mortality within 8 and 11 days, respectively (Ahne *et al.*, 1990). Adult fish are also susceptible, but mortality does not exceed 30% (Ahne *et al.*, 1991). Histopathology and electron microscopy in experimentally infected sheatfish of 4–5 cm revealed alterations in all organs (Ogawa *et al.*, 1990). Endothelium and reticuloendothelial cells are the main target. Periarteriolar necrosis of the haematopoietic tissue and degeneration of tubular and duct epithelia are prominent in kidneys. Gill epithelium and chloride cells are hyperplastic and oedematous. The lumen of the circulatory system is congested and the proliferating cells contain eosinophilic inclusions. Alterations in the skin include proliferation and necrosis of epithelial cells, hyperplasia of monocellular glands and zonal haemorrhage in hypodermis. Myocarditis and endocarditis are diffuse. Small necrotic foci are seen in the liver and spleen. Glial proliferation and spongiosis in the brain are also pronounced. The pathology and incubation most closely resemble those of EHN.

The black bullhead iridovirus disease caused by ECV can induce total acute mortality in pond populations and is highly lethal to experimentally infected subadults and adults. Loss of appetite is evident 1–2 days before the onset of clinical signs. Other signs include oedema and haemorrhage around pectoral and pelvic girdles and in internal organs. The kidney is the principal target organ and both the haematopoietic and the excretory part are severely altered. Blood vessel walls are damaged. Necrosis in the spleen and kidney can be severe. Interlamellar spaces in gills are obliterated and lamellar fusion is evident (Pozet *et al.*, 1992).

One survivor of ECV infection had neutralizing antibodies (Pozet *et al.*, 1992). However, rabbits react to these viruses by producing antibodies suitable for IFAT and ELISA.

The disease caused by LMBV occurs during the summer and predominantly affects largemouth bass over 30 cm in length (Grizzle and Brunner, 2003). Fish lose equilibrium, are found floating at the surface, typically with lesions of the swim bladder, but often have no other grossly visible lesions (Plumb *et al.*, 1996). Thick, yellow or brown exudate was found in the swim bladder of the fish collected a few weeks after die-off (Hanson *et al.*, 2001). Experimental injection with LMBV into largemouth bass showed severe inflammation in the abdominal cavity, displayed spiral swimming and then lethargy (Zilberg *et al.*, 2000), which is consistent with distribution of the virus in experimentally infected fish (Beck *et al.*, 2006). Antibodies develop in fish exposed to LMBV, and the immune response following exposure to the virus may reduce the likelihood of the disease or reduce the severity of the disease in subsequent years (Grizzle and Brunner, 2003).

SGIV causes sleepy grouper disease (SGD) and extreme lethargy in affected grouper (*E. tauvina*), with few visible external signs (Chua *et al.*, 1994). Mortalities occurred either gradually over the week from the onset of clinical signs, or over a shorter period and in large numbers if fish were stressed. Consistent tissue changes were found in the spleen, heart and kidney of affected fish and included disruption of ellipsoid sheaths, necrosis of splenic pulp with pyknosis and karyorrhexis. Basophilic intranuclear inclusion bodies were observed and circumscribed bodies of eosinophilic granular to amorphous material often

appeared to be encapsulated by epitheloid-type cells.

Control, treatment and epizootiology

Natural epizootics of EHN occur in the early summer among young redfin perch and last for 2–3 weeks, but are also recurrent in several major waterways in Victoria, Australia (Langdon, 1989). Temperature dependency of the EHNV infection in redfin perch was confirmed by experimental infection and disease did not occur at temperatures below 12°C (Whittington and Reddacliff, 1996). Adults are rarely affected. One hundred-day-old survivors of disease outbreaks are resistant to challenge (Langdon, 1989). Redfin perch carriers were not detected and there was no evidence of vertical transmission, although an unknown reservoir and carrier host were suspected. Silver gulls (*Lams novaehollandiae*) and great cormorants (*Phalacrocorax carbo*) can spread EHNV by the regurgitation of ingested material (Whittington *et al.*, 1996). Other means of spread include fish transportation, transfer on boats, nets and other equipment, as well as water flow and migration of carrier fish in a catchment area (Whittington *et al.*, 1996).

The close relatedness of fish and amphibian ranaviruses and the pathogenicity of Bohle iridovirus for barramundi should be kept in mind in programmes for avoiding pathogens in aquaculture (Hedrick *et al.*, 1992; Ahne *et al.*, 1993). Frogs are ubiquitous on large farms of warmwater fish and could represent reservoirs and vectors of fish pathogens. Hedrick *et al.* (1992), Hedrick and McDowell (1995) and Hedrick (1996) consider transcontinental movements of amphibians and exotic ornamental fish as possible reasons for the appearance of similar viruses in Australia and Europe. It has been suggested that control be extended to aquatic amphibians and tropical aquarium fish (Ahne *et al.*, 1993; Hedrick, 1996). Australia enforces stringent quarantine restrictions on the import of ornamental fish and restriction of sale and movement of rainbow trout from EHN-affected hatcheries (Doyle *et al.*, 1996). Only general preventive measures are applicable for diseases in this group. More data on epizootiology of these diseases may lead to better targeting of control measures.

Fish mortality due to LMBV infections have occurred in some wild populations, but infected fish are also found in many locations where there have been no reports of die-offs (Plumb *et al.*, 1999; Grizzle *et al.*, 2003). There are no reports of effects on wild fish populations. Variation in virulence could result from genetic differences in the virus (Goldberg *et al.*, 2003) or the fish, and environmental stress such as water quality and fish density could also be important in the initiation of disease outbreaks (Grizzle and Brunner, 2003; Inendino *et al.*, 2005). Grant *et al.* (2005) indicated that angling itself might have minimal effects on the survival of largemouth bass infected with LMBV but that angling-related practices that place infected fish and uninfected fish together in a limited water volume might facilitate viral transmission.

EHNV is extremely resistant to drying and can survive for months in water (Langdon, 1989). The virus is susceptible to 70% ethanol, 200 mg/l sodium hypochlorite or heating to 60°C for 15 min. Ultraviolet sterilizing units are effective in neutralizing EHNV at flow rates used in hatcheries (Miocevic *et al.*, 1993). Effective concentrations of commercially available disinfectants against amphibian ranavirus for 1 min exposure are: Nolvasan®, 0.75%; bleach, 3.0%; and Virkon S®, 1.0% (Bryan *et al.*, 2009).

Conclusions and recommendations for future studies

Amphibian ranavirus has been listed in the OIE Aquatic Animal Health Code since 2008 and a number of papers describing occurrence of ranavirus infection of amphibian and reptile have been published. Significance of amphibian ranavirus to wild and aquaculture fish species, and also that of fish ranavirus to amphibian animals, should be defined and, accordingly, the taxonomic relationship should be resolved. Since ranavirus is implicated in losses of wild fish stocks like cases of LMBV infection, the role and kinetics of ranaviruses and their

infections in aquatic animal communities should be made clearer. The transmission mechanism of SGIV and GIV and the interaction between host and virus remain to be defined and control measures including vaccine for SGIV and GIV infection in grouper aquaculture operation should be developed, because groupers are a promising aquaculture species in South-east Asian countries.

Red sea bream iridoviral disease (RSIVD)

The disease agent

Red sea bream iridovirus (RSIV; Inouye *et al.*, 1992), the causative agent of red sea bream iridoviral disease (RSIVD) in marine fish, is classified currently in the genus *Megalocytivirus*, which consists of one species with the type strain infectious spleen and kidney necrosis virus (ISKNV) isolated from mandarin fish (*Siniperca chuatsi*) cultured in China (He *et al.*, 2000; Chinchar *et al.*, 2005). Megalocytiviruses can be subdivided in three groups by phylogenetic analysis of the major capsid protein (MCP) gene (Nakajima and Kurita, 2005): ISKNV group from mostly freshwater and brackishwater fish, RSIV group from marine fish and TRBIV group mostly from flatfish, including Japanese flounder, which is consistent with analysis by Wang *et al.* (2007). OIE (2006) mentions that RSIVD is also caused by viruses of the ISKNV group, but has suspended the conclusion that TRBIV group viruses are implicated in RSIVD.

Megalocytiviruses have been detected from a range of grouper species (*Epinephelus* spp.) cultured in Taiwan (tiger grouper iridovirus [TGIV]: Chou *et al.*, 1998; Wang *et al.*, 2009), Thailand (Danayadol *et al.*, 1997; Kasornchandra and Khongpradit, 1997) and Singapore (grouper sleepy disease iridovirus [GSDIV]: Sudthongkong *et al.*, 2002a; Gibson-Kueh *et al.*, 2004). The viruses were detected from rock bream (*O. fasciatus*) (rock bream iridovirus [RBIV]: Jung and Oh, 2000; Kim *et al.*, 2002, 2005), rockfish (*Sebastes schlegeli*), flounder (*Paralichthys olivaceus*) and sea perch (*Lateolabrax* sp.) (Jeong *et al.*, 2003, 2006; Do *et al.*, 2005) cultured in Korea; mullet (*Mugil cephalus*) cultured in Singapore (Gibson-Kueh *et al.*, 2004); large yellow crocker (*Larimichthys crocea*) (Chen *et al.*, 2003), red drum (*Sciaenops occelayus*) (Weng *et al.*, 2002) and turbot (turbot reddish body iridovirus [TRBIV]: Shi *et al.*, 2004; Kim *et al.*, 2005) cultured in China; and sea bass in the South China Sea (sea bass iridovirus [SBIV]: Sudthongkong *et al.*, 2002a). Also, the viruses affected a range of ornamental fish species including gourami (*Colisa* spp. and *Trichogaster* spp.) (Anderson *et al.*, 1993; Fraser *et al.*, 1993; Paperna *et al.*, 2001; Sudthongkong *et al.*, 2002b), swordtails (*Xiphophorus helleri*) (Paperna *et al.*, 2001), platies (*X. maculatus*) (Paperna *et al.*, 2001), angelfish (*Pterrophllum scalare*) (Rodger *et al.*, 1997), orange chromides (*Etroplus maculatus*) (Armstrong and Ferguson, 1989), Siamese fighting fish (*Betta splendens*) (Gibson-Kueh *et al.*, 2003) and African lampeye (*Aplocheilichthys normani*) (Sudthongkong *et al.*, 2002b) and freshwater fish, Murray cod (*Maccullochella peelii peelii*) cultured in Australia (Lancaster *et al.*, 2003; Go *et al.*, 2006). It is noted that the name 'grouper iridovirus' should be classified particularly carefully because a grouper iridovirus (GIV) isolated from diseased *E. awoara* in Taiwan and Singapore grouper iridovirus (SGIV) belonged to the ranaviruses (Murali *et al.*, 2002; Lai *et al.*, 2003). Reviews of RSIVD are presented by Nakajima *et al.* (2001) and Nakajima and Kurita (2005).

RSIV has an unenveloped-icosahedral virion ranging from 200 to 240 nm in diameter, containing a dense core of 120 nm (Inouye *et al.*, 1992). RSIV replicates in several fish cell lines including grunt fin (GF), BF-2, FHM, RTG-2 and CHSE-214 at optimal temperatures of 20–25°C and causes a characteristic enlargement of infected cells. Even the most suitable GF cells give highest virus titre, but it is still at a 10^5 $TCID_{50}$/ml level at most (Nakajima and Sorimachi, 1994). A cell line, hirame natural embryo cells (HINAE), can support viral growth (Kasai and Yoshimizu, 2001; Lua *et al.*, 2005). Virus isolation is still not easy, although some trials of developing marine fish cell line are more

susceptible to megalocytivirus (Imajoh *et al.*, 2007; Wen *et al.*, 2008). Recently, a cell line MFF-1 from a mandarin fish fry stably susceptible to ISKNV has been reported (Dong *et al.*, 2008).

Serotypes of RSIV have not been reported to date. Polyclonal anti-RSIV serum shows cross-reactivity with ESV and EHNV infected cells, whereas monoclonal anti-RSIV antibodies against structural or induced viral protein react only with RSIV infected cells (Nakajima *et al.*, 1998). Based on amino acid sequence analysis of the *MCP* gene, megalocytiviruses show a high level of 93% identity (Jeong *et al.*, 2003; Chinchar *et al.*, 2005).

Geographical distribution and host range

RSIV and ISKNV group viruses have been reported in East and South-east Asian countries including China, Chinese Taipei, Hong Kong, Japan, Korea, Malaysia, Philippines, Singapore and Thailand. TRBIV group viruses have been reported in China and Korea (OIE, 2006).

Hosts of RSIV have been reported from 31 marine-cultured species in Japan: 28 species from order *Perciformes*, two species from order *Pleuronectiformes* and one species from order *Tetraodontiformes* (Kawakami and Nakajima, 2002). OIE (2006) has listed 40 marine species susceptible to RSIV and four marine species susceptible to ISKNV. Experimental transmission of ISKNV showed smallmouth bass (*Micropterus salmoides*) and grass carp (*C. idella*) were susceptible to ISKNV (He *et al.*, 2002b), and an ISKNV group virus from pearl gourami was pathogenic to a marine fish, rock bream (Jeong *et al.*, 2008), other than the OIE host list.

Economic importance of the disease

RSIVD has caused significant losses of aquaculture fish since its first occurrence in 1990, and the affected fish are not only red sea bream but also 30 other marine fish species ranging from juvenile to marketable size in Japan (Matsuoka *et al.*, 1996; Nakajima *et al.*, 2001; Kawakami and Nakajima, 2002; Nakajima and Kurita, 2005). Because of their economic impacts on aquaculture, licensed vaccines have been used for marine aquaculture in Japan. In Taiwan, the disease causes 50–90% mortality of red sea bream and significant losses of grouper in net-cage culture (Chao *et al.*, 2002; Wang *et al.*, 2003). In Korea, the rock bream (*O. fasciatus*) is reported as highly susceptible to RSIVD. High mortality in net-caged rock bream occurred in 1998, and RSIV has been the major culprit of mass mortality of rock bream, reaching over 90% in Korea (Jung and Oh, 2000; Jeong *et al.*, 2006; Kim *et al.*, 2008).

Diagnostic methods

Specific diagnosis of RSIVD is generally based on the direct detection of RSIV or ISKNV from the affected fish tissues (OIE, 2006), and current diagnostic procedure is referred to the latest OIE manual.

Since the virus isolation on cell culture is still not a reliable method, IFAT using specific monoclonal antibody (M10 MAb) and PCR are available for detection (Nakajima and Sorimachi, 1995; Nakajima *et al.*, 1995; Kurita *et al.*, 1998). Based on the abundance of infected cells, it is recommended that the spleen and/or kidney of affected fish be sampled. In virus isolation of RSIV, grunt fin (GF) cell line (Clem *et al.*, 1961) is used and a routine virus isolation procedure with an incubation temperature at 25°C for 10 days is employed. The CPE is characterized with initially round, enlarged and balloon-like cells finally detached from the bottom. Identification of virus in cell culture is based on IFAT using M10 antibody or PCR (OIE, 2006). Direct detection from fish tissues is carried out by IFAT on a smear specimen or PCR. IFAT on a smear specimen with M10 MAb is a simple, rapid and reliable method for routine diagnosis in mostly overt infection cases. PCR and nested-PCR methods have been reported (Kurita *et al.*, 1998; Oshima *et al.*, 1998; He *et al.*, 2001; Chao *et al.*, 2002; Jeong *et al.*, 2003, 2004, 2006), and a PCR method in the OIE manual detects both RSIV and ISKNV. A real-time PCR assay and LAMP for the detection of RSIV have been developed (Caipang *et al.*, 2003, 2004). Since, in the

case of persistent infection of RSIV, the virus detection rate by nested-PCR is low in the spleen compared with other organs such as the heart and intestine, it is recommended that not only the spleen but also other organs should be used to diagnose persistent infection (Choi *et al.*, 2006). Fish antibodies induced against rock bream iridovirus (RBIV) infection were detected using ELISA with recombinant viral MCP as an antigen in naturally infected rock bream (Kim, T.J. *et al.*, 2007).

Genome structure and transcription

Megalocytiviruses have a single linear dsDNA molecule. The complete genome sequences of five megalocytiviruses have been reported: RSIV, 112,414 bp (Kurita *et al.*, 2002); ISKNV, 111,362 bp (AF371960; He *et al.*, 2001); RBIV, 121,080 bp (AY532606; Do *et al.*, 2004); orange-spotted grouper iridovirus (OSGIV), 112,636 bp (AY894343; Lu *et al.*, 2005); large yellow croaker iridovirus, 111,760 bp (AY779031; Ao and Chen, 2006). Thus, the unit genome size for megalocytivirus is approximately 112–121 kbp with a G+C content of around 54%. As with other members of the family, genomic DNA is permuted circularly, terminally redundant and highly methylated (Chinchar *et al.*, 2005).

Sequence analysis of RSIV, ISKNV, RBIV and OSGIV predicted respective 116, 124, 118 and 121 ORFs with coding capacities ranging from approximately 40 to 1200 amino acids. Annotation study showed the presence of predicted 116–117 ORFs in the genome (Eaton *et al.*, 2007).

Transcription of iridoviral DNA is a coordinated sequential process involving the production and regulation of mRNAs that can be classified into immediate-early (IE), delayed-early (E) and late (L) genes according to their temporal synthesis on infection (see Williams, 1996; Chinchar, 2002; Chinchar *et al.*, 2005; Williams *et al.*, 2005). Lua *et al.* (2005) demonstrated that the gene expression of RSIV occurred in a temporal kinetic cascade with these three stages, and 87 transcripts were classified into nine of IE, 40 of E and 38 of L.

Pathogenesis and immunity

Megalocytivirus infection is characterized with the appearance of enlarged cells that stain dark with Giemsa in the spleen, heart, haematopoietic tissue, gills and digestive tract (Fig. 5.23).

The first outbreak of RSIVD among cultured red sea bream was observed in Japan in 1990 (Inouye *et al.*, 1992). Subsequently, the disease has caused mass mortality of cultured marine fish, not only red sea bream but also many other species in the summer season at water temperatures of 25°C and above (Matsuoka *et al.*, 1996; Nakajima and Maeno, 1998; Kawakami and Nakajima, 2002; Jun *et al.*, 2009). Most of the affected fish are juvenile, but mortality of marketable size fish has been reported (Kawakami and Nakajima, 2002). The affected fish were lethargic and exhibited severe anaemia, petechiae of the gills, enlargement of the spleen and petechiae of visceral organs (Inouye *et al.*, 1992). Typically, enlarged cells of about 20 μm diameter characterized by basophilic stainability and Feulgen staining positive reaction were observed in the gills, heart, kidney, liver and spleen. The enlarged cell may originate in infected leucocyte (Inouye *et al.*, 1992) and ultrastructural features of the cells of a grouper have been described in detail (Chao *et al.*, 2004; Mahardika *et al.*, 2004, 2008). Most prominent histopathological changes are observed in the spleen. Infected splenocytes are markedly necrotized.

Experimental infection trials in red sea bream using isolated RSIV at 25°C showed that the disease course was acute and mortality appeared initially at 5 days after inoculation and reached over 50% within 10 days after inoculation (Nakajima *et al.*, 1997), and a similar disease course was obtained in experimental infection of a grouper *E. malabaricus* (Sano *et al.*, 2002). The transcriptional profile of RSIV in red sea bream was constant with the disease course (Lua *et al.*, 2007). The predisposing environmental factor is water temperature and outbreaks have been found in the summer season at about 25°C or higher. In the case of experimental infection of rock bream

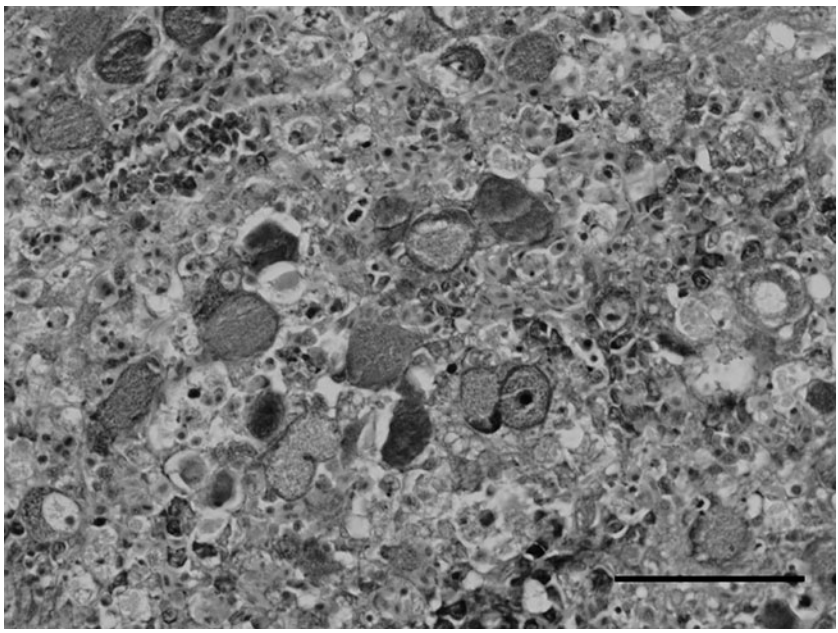


Fig 5.23. Tissue sections of the spleen of red sea bream infected with RSIV showing the appearance of enlarged cells. Giemsa stain. Bar, 50μm.

with RBIV, infections at 13°C resulted in 0% mortality, while those at 25°C caused 100%. This may implicate viral replication ability (Jun *et al.*, 2009). A similar result was obtained from ISKNV infected *Siniperca chuatsi* (He *et al.*, 2002b). Recently, a zebrafish (*D. rerio*) model of ISKNV infection has been reported as a valuable tool for studying the interactions between the virus and its host (Xu *et al.*, 2008).

Control, treatment and epizootiology

The principal mode of transmission is horizontal. There is no evidence of vertical transmission in hatchery operations in Japan. Kim, J.H. *et al.* (2007) described the importance of trash fish harbouring the virus for feeding to cultured fish. An epizootical investigation using nested-PCR in Korea revealed that prevalence of infection in farmed fish obtained from market was 79.7% for rock bream, 31.3% for rockfish (*S. schlegeli*), 27.3% for sea perch (*Lateolabrax* sp.) and 13.2% for red sea bream (Jeong *et al.*, 2006).

A formalin-killed vaccine showed a significant effectiveness in red sea bream under both experimental and field conditions via i.p. injection (Nakajima *et al.*, 1997, 1999). Experimental vaccine studies demonstrated that this vaccine was effective to RSIV infections of yellow tail (*Seriola quinqueradiata*), amberjack (*S. dumerili*), kelp grouper (*E. moara*), striped jack (*P. dentex*), spotted parrot fish (*O. punctatus*) (Nakajima *et al.*, 2002) and to megalocytivirus infection of humpback grouper (*C. altivelis*) (Mahardika *et al.*, 2008). This vaccine has been licensed in Japan since 1999 and is available for red sea bream, malabar grouper (*E. malabaricus*) and fish species belonging to the genus *Seriola*. Caipang *et al.* (2006) and Kim *et al.* (2008) used a recombinant MCP as a vaccine for red sea bream and rock bream, respectively.

Tanaka *et al.* (2003) investigated the effect of fasting to RSIVD in red sea bream. In field trials, a fasting group (fasting for 16 days) showed significantly lower mortality than a feeding group (once daily at almost satiation) for 37 days during September to October. Rearing density of red sea bream

can also be linked to the severity of RSIVD (Tanaka and Inoue, 2005). In a field trial, red sea bream were reared in a neighbouring net pen at three densities of 0.8, 2.8 and 9.6 kg/m^3 for 73 days from mid-July to late September, and the mortality due to RSIVD was 9.3, 32.0 and 47.2% in the respective group. The same density effect was observed in experimentally infected fish reared in an aquarium. The most usual rearing density for red sea bream culture is 4–10 kg/m^3, and the lower rearing density can be practically effective in reducing fish losses due to RSIVD.

RSIV is susceptible to 70% ethanol and heating at 55°C for 30 min (Nakajima and Sorimachi, 1994). Disinfectant efficacies against ISKNV determined with bioassay are: potassium permanganate, 100 mg/l for 15 min; formalin, 2000 mg/l for 15 min; sodium hypochlorite, 200 mg/l for 15 min (He *et al.*, 2002b).

Conclusions and recommendations for future studies

Virus classification and taxonomy for megalocytiviruses should be elucidated. Although the pathognomonic feature of megalocytivirus infection is the appearance of basophilic enlarged cells, even if there is one species in the genus, pathogenicity of viruses from freshwater fish, from brackishwater fish and from marine fish to fish in different environments has to be defined to be categorized. Jeong *et al.* (2008) demonstrated that an iridovirus from freshwater ornamental fish caused mortality of a marine fish, rock bream, in experimental infection, whereas ISKNV experimental infection did not generate any mortality in marine species (He *et al.*, 2002b). A megalocytivirus from a marine fish, spotted knifejaw (*O. punctatus*), was virulent to a freshwater fish, mandarinfish, from which ISKNV was isolated originally in experimental infection (Dong *et al.*, 2010). Since ISKNV group viruses have been detected from marine fish, the relationship between viruses originated in marine and freshwater environments is needed in addition to investigate. Moreover the transmission mechanism and kinetics of RSIV in marine environment are subjects for further investigation.

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6 Salmonid Alphaviruses

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Introduction

Salmonid alphaviruses (SAV) are recognized as serious pathogens of farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss* [Walbaum]) in Europe. Salmon pancreas disease virus (SPDV or SAV subtype 1 [SAV 1]) was the first to be isolated and shown to be the causative agent of pancreas disease (PD) in farmed Atlantic salmon in Ireland and Scotland (Nelson *et al.*, 1995; Rowley *et al.*, 1998; Weston *et al.*, 1999; Welsh *et al.*, 2000). More recently, four further SAV subtypes associated with PD outbreaks have been described in Ireland, Scotland and Norway (Hodneland *et al.*, 2005; Weston *et al.*, 2005; Fringuelli *et al.*, 2008).

Sleeping disease (SD) is an infectious disease of rainbow trout reared in fresh water. The viral aetiology of SD has been suspected for some time (Boucher *et al.*, 1994) and it was confirmed by the isolation of sleeping disease virus (SDV) (Castric *et al.*, 1997), which was characterized as an atypical alphavirus of the family *Togaviridae* and is now known as SAV subtype 2 (SAV 2) (Villoing *et al.*, 2000a). SAV 2 has been isolated recently from diseased rainbow trout in England and Scotland (Branson, 2002; Graham *et al.*, 2003a) and also has been identified in Germany (Bergmann *et al.*, 2005). Infection with SAV 2 has also been shown to be present in rainbow trout in fresh water in Italy and Spain (Graham *et al.*, 2006). Subtype 2 has also been isolated recently from marine farmed Atlantic salmon in Scotland (Fringuelli *et al.*, 2008).

The main pathological lesions are similar in PD and SD, with extensive loss of pancreatic acinar cells during the viraemic phase, concurrent cardiomyocytic degeneration and inflammation and subsequent skeletal muscle degeneration and fibrosis (Munro *et al.*, 1984; Ferguson *et al.*, 1986a; Rodger *et al.*, 1991; Boucher and Baudin Laurencin, 1994; McLoughlin *et al.*, 2002; Taksdal *et al.*, 2007). Outbreaks of PD have been described in seawater only, but SAV has been detected using RT-PCR and confirmed by sequencing in Atlantic salmon suffering from haemorrhagic smolt syndrome (HSS) in fresh water (Nylund *et al.*, 2003; Karlsen *et al.*, 2005).

The epizootiology of salmonid alphaviral diseases (SAD) is complex and the outcome of infection depends on fish age, species and strain, site characteristics, water temperature and stressors (Wheatley *et al.*, 1995; Crockford *et al.*, 1999; Rodger and Mitchell, 2007). In the past 5 years, PD has re-emerged as a major source of losses due to mortality and growth reduction in farmed Atlantic salmon at any stage during their marine production cycle (McLoughlin *et al.*,

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2003). A review of salmonid alphavirus was published in 2007 (McLoughlin and Graham, 2007) and this chapter extends that review, covering all known information on this new group of economically important viruses in salmonids.

History, Geographical Distribution, Epizootiology and Economics of PD and SD

Various descriptions of PD in farmed Atlantic salmon have been recorded since it was first recognized in Scotland in 1976 (Munro *et al.*, 1984; Ferguson *et al.*, 1986a,b; McVicar, 1987, 1990; Rodger *et al.*, 1994). The first descriptions in Ireland were published in the early 1990s (Rodger *et al.*, 1991; Murphy *et al.*, 1992). The initial study of PD in Norway was published by Poppe *et al.* (1989) and, more recently, a full description of PD in Atlantic salmon and sea-reared rainbow trout has been published by Taksdal *et al.* (2007). PD was described only once in North America in 1987, but no virus was detected (Kent and Elston, 1987), and this remains the only report of a PD-like condition outside Europe. A dual infection of infectious salmon anaemia (ISA) virus and a toga-like virus was described in New Brunswick, Canada, in 2000 (Kibenge *et al.*, 2000), but no further information on the identity of this togavirus has been published.

Initially, the disease was usually described in Atlantic salmon smolts during their first year at sea, with peak prevalence occurring from late July to early September (McVicar, 1987; Crockford *et al.*, 1999), but in recent years outbreaks have occurred at all stages of the marine production cycle of Atlantic salmon (Rodger and Mitchell, 2007). Indeed, reports from Scotland and Norway indicate that PD is most commonly diagnosed in grower fish during their second year at sea (T. Turnbull and O. Breck, personal communication, 2006). Mortality rates in PD outbreaks in Ireland have ranged from 1 to 48% (Wheatley, 1994; Menzies *et al.*, 1996; Crockford *et al.*, 1999; McLoughlin *et al.*, 2003; Rodger and Mitchell, 2007). Crockford *et al.* (1999) reported that mortality rates varied significantly between sites, but they did not vary significantly between years on the same site, indicating a complex multifactorial aspect to this disease. High mortalities tend to occur in higher energy sites, i.e. those with fast currents or oceanic location, and when the fish are returning to feeding after a period of inappetence (Rodger *et al.*, 1991; Crockford *et al.*, 1999). In 2003–2004, the Irish salmon industry estimated that PD resulted in a loss of turnover of €35 million, with a €12 million loss of profit (Ruane *et al.*, 2008).

Variable mortality levels have been attributed to PD in other countries, with recent reports of significant PD-related mortalities in Norway, where PD has spread north and south of the original affected area around Bergen (Karlsen *et al.*, 2005; Taksdal *et al.*, 2007). Economic losses from PD in Norway have been estimated at NOK10 million/500,000 fish on a farm (Aunsmo *et al.*, 2005). There has been no recent published information on the prevalence and impact of PD in Scotland, but individual marine sites have reported significant losses. PD is endemic in most salmon marine sites in Ireland (Rodger and Mitchell, 2007) and in historically infected sites in other countries, and outbreaks tend to recur in each successive generation of fish introduced on to the site, irrespective of the duration of the fallowing period (McLoughlin *et al.*, 2003). This suggests a substantial reservoir of infection in the seawater environment, or a freshwater carrier state (Karlsen *et al.*, 2005). In 2005, PD was recognized in regions both north and south of the previously identified endemic area around Bergen and in the far north of Norway, strongly suggesting horizontal transmission (Karlsen *et al.*, 2005). There are some indications that SAV 3 may be present at low levels in small numbers of salmon fry in fresh water and salmon broodstock in Norway, but the significance of these findings remains uncertain (Karlsen *et al.*, 2005; Nylund, 2007). There is no evidence to date of clinical PD or seroconversion to SAV in wild freshwater or marine

salmonids (Graham *et al.*, 2005). There is some evidence that SAV 2 can be transmitted vertically from rainbow trout broodstock to eggs and fry following intraperitoneal (i.p.) challenge, but further work is required to confirm this observation (Castric *et al.*, 2005).

The age and type of the fish, i.e. smolt type (S0 or S1), and fish strain, may also influence the severity of PD infection (Crockford *et al.*, 1999; McLoughlin *et al.*, 2006). The sea temperature at the time of infection may also have some bearing on the pathogenesis of PD, with more acute and shorter-lived outbreaks occurring between 8 and 15°C and more chronic outbreaks when temperatures are low (< 8°C) (M.F. McLoughlin, unpublished observations, 2006). Up to 15% of survivors failed to grow and became runts (Munro *et al.*, 1984). The average duration of PD outbreaks in Ireland in the early 1990s was 112 days (SE=7.7, n=37) (Crockford *et al.*, 1999), and in 2002, the average duration of a PD outbreak increased to 141 days, with a range of 61–288 days (n=13) (McLoughlin *et al.*, 2003).

Recently, Graham *et al.* (2007a) have described the first detailed report of subclinical (no clinical signs, no significant mortalities and relatively mild histopathological lesions) SAV infection in marine-reared Atlantic salmon in Scotland. This suggests that other factors related to the environment, host and/or pathogen might have the potential to influence the severity of a PD outbreak in a given population.

Sleeping disease (SD) is a serious infectious disease of farmed rainbow trout reared in fresh water in Europe. This disease was first observed in France (Boucher and Baudin Laurencin, 1994) and it affects rainbow trout at all stages of production. SD is endemic in parts of France, affecting 30–40% of production (Boucher *et al.*, 1994; Villoing *et al.*, 2000a; Bremont, 2007).

More recently, SD has been reported in England and Scotland, where SAV 2 was isolated from affected fish (Branson, 2002; Graham *et al.*, 2003a), and in Germany (Bergmann *et al.*, 2005). Clinical disease has also been reported from Italy and Spain and the involvement of SAV 2 confirmed using serology, virology and RT-PCR (Graham *et al.*, 2007c). Mortality levels have been very variable, from negligible to over 22% in affected populations (Boucher and Baudin Laurencin, 1994; Graham *et al.*, 2003a). The current geographical distribution of known SAV subtypes and the farmed species from which they have been isolated are summarized in Table 6.1. Brown trout (*S. trutta*) have also been shown to be susceptible to infection under experimental conditions (Boucher *et al.*, 1995), and evidence of infection has also been found in sea trout (*S. trutta*) and Arctic charr (*Salvelinus alpinus*) in Norway (Nylund, 2007).

The Disease Agent

Current classification

The genus *Alphavirus* within the family *Togaviridae* has been reviewed extensively (Weaver *et al.*, 2000; Powers *et al.*, 2001). Prior to the isolation of alphaviruses from salmonids, members of the genus already contained at least 40 members and were found on all continents, including Antarctica (Linn *et al.*, 2001). Restrictions on the geographic distribution of individual species were recognized to reflect the ecological requirements and availability of both suitable vector species and reservoir hosts. All of the alphaviruses shared a common epidemiology, being transmitted between primary avian or mammalian reservoir hosts by haematophagous arthropod (typically mosquito) vectors, although lice and ticks have also been implicated (Gresikova *et al.*, 1978; Linthicum and Logan, 1994; Linn *et al.*, 2001). Serological and phylogenetic analysis of existing members of the genus Alphavirus resulted in their being assigned to at least seven antigenic complexes and three main geno-complexes comprising the VEE-EEE group (Venezuelan eastern encephalitis-Eastern equine encephalitis), the SF group (Semliki Forest) and the Sindbis (or

Table 6.1. The current geographical distribution of known SAV subtypes in farmed salmonids and the species and production stage from which they have been isolated.

SAV subtype and source	Alternative virus name	Location	Species	Disease	References
SAV 1 Marine only	SPDV	Ireland Scotland	Atlantic salmon	PD	Nelson *et al.* (1995) Rowley *et al.* (1998) Weston *et al.* (2005)
SAV 2 Fresh water and marine	SDV	France, UK, Spain, Italy, Germany	Rainbow trout Atlantic salmon	SD and PD	Boucher *et al.* (1994) Branson (2002) Graham *et al.* (2007c) Fringuelli *et al.* (2008)
SAV 3 Marine and fresh water	NSAV	Norway	Atlantic salmon Rainbow trout	PD	Christie *et al.* (1998) Hodneland *et al.* (2005)
SAV 4 Marine	SPDV	Ireland and Scotland	Atlantic salmon	PD	Fringuelli *et al.* (2008)
SAV 5 Marine	SPDV	Scotland	Atlantic salmon	PD	Fringuelli *et al.* (2008)
SAV 6 Marine	SPDV	Ireland	Atlantic salmon	PD	Fringuelli *et al.* (2008)

WEE [Western equine encephalitis]) group (Weaver *et al.*, 2000; Powers *et al.*, 2001). The recently described southern elephant seal virus (SESV) is considered to be a member of the Semliki Forest clade, whereas the salmonid alphaviruses represent a new clade within the genus (Leurs *et al.*, 2005). Viruses can also be grouped according to their predominant geographical distribution, with the VEE-EEE being exclusively New World, whereas the SF and Sindbis groups are predominantly found in the Old World (Weaver *et al.*, 2000; Linn *et al.*, 2001). Many of the Old World viruses produce fever, rash and arthralgia in humans, whereas the New World viruses frequently result in encephalitis. A number of the alphaviruses have different species of birds as their primary vertebrate reservoir, but are typically also able to replicate in mammals, whereas others have a primary mammalian vertebrate reservoir. The latter viruses tend to be genetically diverse, with non-overlapping distributions, whereas the former tend to be less diverse with greater geographic ranges, due to the higher degree of host mobility (Powers *et al.*, 2001). Linn *et al.* (2001) have proposed an alternative classification of the alphaviruses based on their use of mammals or birds as their primary enzootic reservoir host. In this classification, the recently described alphaviruses from salmonids therefore represent a distinct third grouping.

It has been suggested that the alphaviruses originated from insect-borne plant viruses in the New World, with at least three subsequent transoceanic introductions between the hemispheres spread by migratory birds (Powers *et al.*, 2001). Phylogenetic studies consistently place the salmonid alphaviruses at the base of the alphavirus tree, in line with an ancestral alphavirus adapting to fish early in the development of the genus.

The first reported isolation of an alphavirus from salmonids was by Nelson *et al.* (1995), who described the isolation of a virus from Atlantic salmon showing clinical signs of pancreas disease. They showed that the virus was capable of reproducing PD experimentally and consequently named the agent salmon pancreas disease virus (SPDV). Shortly afterwards, Castric *et al.* (1997) reported the first isolation of the causal agent of SD, for which they proposed the name sleeping disease virus (SDV). Further physico-chemical, biochemical and morphological

studies of SPDV and SDV (Nelson *et al.*, 1995; Villoing *et al.*, 2000a) demonstrated both to be spherical RNA viruses with a diameter of 65.5±4.3 nm and surrounded by a double-layered lipid envelope containing spikes. They were shown to be sensitive to chloroform, rapidly inactivated at pH 3.0, stable up to 37°C and with a buoyant density in caesium chloride of 1.20 g/ml. Based on these properties, both SPDV and SDV were considered to be toga-like viruses.

Subsequently, their identity as alphaviruses has been confirmed independently by sequencing of the 3′ portion of their genomes. Initially, Weston *et al.* (1999) sequenced a 5.2 kb region of the 3′ end of SPDV. Sequence analysis showed considerable organizational and sequence identity with other alphaviruses with a 26S (approximately 4 kb) subgenomic RNA involved in the synthesis of the structural proteins (Welsh *et al.*, 2000). Predicted sizes of the viral glycoproteins were 438 aa (E2) and 461 (E1), these being larger than those of the other alphaviruses. Partial genome sequence studies of SDV revealed similar findings (Villoing *et al.*, 2000a). By this time, Boucher and Baudin Laurencin (1996) had suggested that PD and SD were caused by similar or identical agents, based on comparative histological studies and the demonstration of acquired cross-protection in experimental infections. This observation was substantiated when the partial E1 gene and entire polyprotein amino acid sequences of SPDV and SDV were compared in a phylogenetic analysis as part of a much larger study on the evolutionary relationships of the alphaviruses (Powers *et al.*, 2001). This placed both viruses in a fish clade at the base of the alphavirus tree, consistent with an early divergence from the mosquito-borne alphaviruses and causing the authors to suggest that SDV was a strain or subtype of SPDV. The identity of both SPDV and SDV as new aquatic alphaviruses, and the close relationship between them, was confirmed in a subsequent collaborative study (Weston *et al.*, 2002) in which the entire genomes of F93–125 and S49P, the reference strains of SPDV and SDV, respectively, were sequenced. This study revealed a strong genetic similarity, consistent with both SPDV and SDV being closely related isolates of the same virus. SPDV and SDV shared 91.1% nucleotide sequence identity over their entire genomes and 95 and 93.6% amino acid identities over their structural and non-structural proteins, respectively. This conclusion was further supported by studies on the biological properties of the viruses, including cross-infection and cross-neutralization trials, which showed only minor biological differences. As a result, it was concluded that SPDV and SDV were closely related isolates of the same viral species, for which the name salmonid alphavirus (SAV) was proposed (Weston *et al.*, 2002).

In 2005, Weston *et al.* and Hodneland *et al.* independently reported the identification of a new subtype of SAV from farmed marine Atlantic salmon and rainbow trout in Norway. Weston *et al.* (2005) showed consistent differences between strains independent of the region of the genome used and proposed that SPDV and related salmonid alphaviruses be termed SAV subtype 1 (SAV 1), SDV-like viruses be termed SAV 2 and the new Norwegian subtypes be termed SAV 3. The findings of Hodneland *et al.* (2005) supported and extended the work of Weston *et al.* (2005), presenting full-length genome sequences for four separate Norwegian viruses (which they termed Norwegian salmonid alphavirus, NSAV). These NSAV/SAV 3 genomes were found to have 91.6 and 92.9% nucleotide similarity to SPDV and SDV, respectively.

Fringuelli *et al.* (2008) have published the most comprehensive phylogenetic study to date, comprising 48 viruses from farmed Atlantic salmon and rainbow trout in the Republic of Ireland, Northern Ireland, Scotland, England, Norway, France, Italy and Spain. This work, based on partial gene sequences from E2 and nsP3, has identified three further SAV subtypes: SAV 4, from Atlantic salmon in Scotland and the Republic of Ireland; SAV 5, from Atlantic salmon in Scotland; and SAV 6, from Atlantic salmon in the Republic of Ireland. A further noteworthy finding in this study was the first report of SAV 2 strains from marine Atlantic salmon. Previously, these strains had been

reported from freshwater rainbow trout only. Consistent with previous studies (Hodneland *et al.*, 2005; Weston *et al.*, 2005), the level of intrasubtype variation was low.

Phenotypic and antigenic characteristics

Although the International Committee on Viral Taxonomy of Viruses (ICTV) has now adopted a polythetic rather than an antigenic definition of viral species (van Regenmortel, 2000), the latter has been used as the basis of *Alphavirus* classification by the Subcommittee on Interrelationships Among Catalogued Arboviruses (SIRACA) of the American Committee on Arthropod-Borne Viruses (Calisher and Karabatsos, 1988). In the latter classification, viruses with fourfold or greater differences in cross-reactivity in both directions were recognized as different viral species, while virus isolates showing a fourfold or greater difference in one direction only were considered to be subtypes of the same virus. Antigenic complexes contain viruses showing greater cross-reactivity to each other than to members of other complexes. Based on this antigenic categorization, all alphaviruses are considered antigenically related to each other. While use of the term 'subtype' to describe the groupings of SAV identified by phylogenetic study is now accepted and used routinely, the precise level of antigenic relatedness between the six subtypes remains to be determined.

Evidence of cross-protection and cross-neutralization between SAV strains has been demonstrated, leading to the suggestion that at least SAV 1, 2 and 3 may represent a single viral serotype (Boucher and Baudin Laurencin, 1996; Christie *et al.*, 1998; McLoughlin *et al.*, 1998; D. Graham, unpublished data). However, caution needs to be exercised in the interpretation of some of these findings, since Fringuelli *et al.* (2008) have shown that the Atlantic salmon-derived SPDV strain P42p used in some of these studies is, in fact, also an SAV 2 strain along with all SDV strains examined to date.

Glycoproteins E1 and E2 (55 and 50 kDa, respectively; Welsh *et al.*, 2000) are considered to be the immunodominant viral proteins, with E2 containing most neutralizing epitopes (particularly between amino acids 170 and 220) and E1 having more conserved, cross-reactive epitopes (Weston *et al.*, 1999; Powers *et al.*, 2001; Moriette *et al.*, 2005). To date, the range of monoclonal antibodies (MAbs) available to investigate the antigenic variability of SAVs is limited, possibly reflecting an apparent low level of antigenicity of SAV in mice (Todd *et al.*, 2001; Moriette *et al.*, 2005). Welsh *et al.* (2000) and Todd *et al.* (2001) described the production of eight MAbs against F93–125, the reference SAV subtype 1 strain, which demonstrated reactivities against the capsid protein and E1 or E2. Subsequently, MAbs have also been raised against S49P, the reference SAV subtype 2 (Weston *et al.*, 2002; Moriette *et al.*, 2005). These MAbs have been used subsequently in a series of studies to examine their reactivity with a range of SAV isolates. Overall, these have shown that MAbs raised to SAV 1 are reactive with isolates from marine farmed Atlantic salmon and rainbow trout in Ireland, Scotland and Norway. In contrast, MAbs raised against S49P, a freshwater trout isolate (SAV 2), were only reactive with other freshwater rainbow trout isolates, with the exception of a single Scottish Atlantic salmon isolate (P42p) (Todd *et al.*, 2001; Weston *et al.*, 2002; Jewhurst *et al.*, 2004; Moriette *et al.*, 2005). This observation is consistent with the recent identification of P42p as an SAV 2 subtype from marine Atlantic salmon, the same subtype to which all sequenced freshwater trout isolates currently belong (Fringuelli *et al.*, 2008). Further studies, using isolates characterized at the molecular level, will be required to investigate fully the antigenic relatedness between different SAV subtypes.

While the level of mortality associated with outbreaks of SAV disease can vary markedly (Menzies *et al.*, 1996; Crockford *et al.*, 1999), viral determinants of virulence remain to be determined. Studies with another alphavirus, Venezuelan equine encephalitis virus, have shown that the titre

to which the virus replicates in the vertebrate host is related to pathogenicity and that only seven amino acid changes in the entire genome (located in nsP1, nsP3, capsid, E2 and the 3′ untranslated region) distinguish enzootic and epizootic strains (Walton *et al.*, 1973; Wang *et al.*, 1999).

Ultrastructure

Alphavirus particles consist of an icosahedral nucleocapsid containing the single-stranded, positive-sense genomic RNA and capsid protein, surrounded by a tight-fitting envelope derived from the host cell plasma membrane containing viral glycoproteins (Strauss and Strauss, 1994; Cheng *et al.*, 1995). The nucleocapsid contains 240 capsid proteins in 30 hexameric and 12 pentameric subunits arranged in a T=4 structure. The highly basic and poorly conserved N-terminal domain of the capsid protein binds viral RNA in the core of the particle, while the conserved C-terminal domain interacts with other capsid proteins and the cytoplasmic domains of envelope glycoproteins. Each virus particle also contains 240 E1 and E2 glycoproteins in the viral envelope. These are arranged as heterodimers, which in turn are further organized into 80 trimers. The C-terminal cytoplasmic domain of each E2 glycoprotein interacts with the capsid proteins of the nucleocapsid in a 1:1 relationship.

Genome Structure and Transcription

The genomic structure and function of the alphaviruses have been described and reviewed previously (Strauss and Strauss, 1994; Weaver *et al.*, 2000). Briefly, alphaviruses have a single-stranded, positive-sense RNA genome of 11–12 kb. The viral RNA has a 7-methyl guanosine cap at the 5′ end and a poly A tail at the 3′ end. The genome is organized into two open reading frames (ORFs) separated by a short non-translated region (Fig. 6.1). The first ORF, comprising the 5′ two-thirds of the genome, encodes four non-structural proteins, termed nsP1, nsP2, nsP3 and nsP4 (the RNA polymerase), which are synthesized as polyprotein precursors with subsequent proteolytic cleavage. All of these proteins perform critical roles in subsequent protein processing and RNA replication (see Strauss and Strauss, 1994).

E2 is considered responsible for binding to receptors on the surface of target cells. Conserved laminin receptors and a heparan–sulfate proteoglycan receptor have been shown to be capable of fulfilling this role, interacting with neutralizing epitopes on E2 between amino acids 170 and 220. The virus particle is internalized by endocytosis, and a reduction in pH within the endosome leads to E1-mediated fusion of the viral envelope and the endosomal membrane, followed by release of the nucleocapsid into the cell cytoplasm. Following release of the viral genome from the nucleocapsid, it is bound by ribosomes, serving directly as messenger RNA. Termination of translation at an opal codon between nsP3 and nsP4 yields P123, whereas read-through of this codon yields polyprotein P1234 containing all four non-structural proteins. Initially, autoproteolytic cleavage of P1234 by the C-terminal half of nsP2 produces a membrane-associated replicase complex of P123, nsP4 and host cell proteins, which results in synthesis of full-length, negative-sense RNA genomes. Further autoproteolytic cleavage of P123 by nsP2 results in release of mature nsP1, nsP2 and nsP3 proteins, which, along with nsP4, function as a positive-sense RNA replicase complex, with only positive-sense full genome (49S) and subgenomic (26S) RNA (the latter encoded by the second ORF) being synthesized thereafter. This second ORF, comprising the 3′ third of the genome, encodes a 26S subgenomic mRNA that ultimately produces a polyprotein containing five structural proteins. Co- and post-translational cleavage of the polyprotein releases the capsid protein, glycoproteins (gp) E3 and E2, the 6K protein and E1. Firstly, serine protease activity of the capsid protein results in its release from the polyprotein. The remaining polyprotein is translocated to the rough endoplasmic reticulum, where carbohydrate moieties are added and further cleavage by signalases

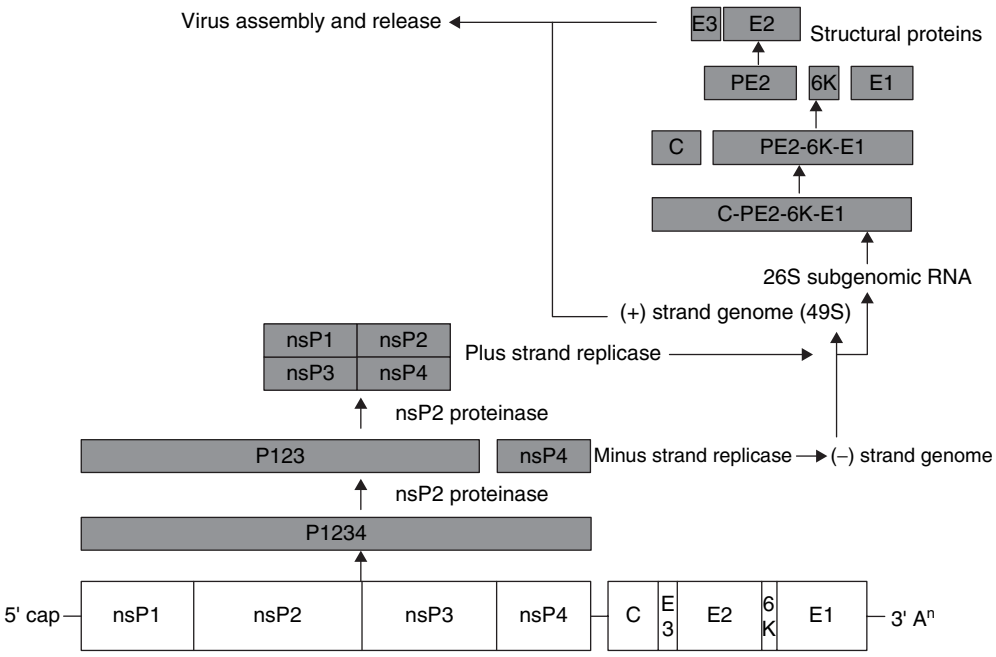


Fig. 6.1. Schematic showing genome organization and replication. The genome consists of two open reading frames, the first encoding the non-structural proteins (nsP1–4) and the second encoding the structural proteins. In infected cells, translation of the non-structural ORF yields a polyprotein P1234 which is cleaved initially by nsP2 proteinase activity to yield the P123 and mature nsP4, which, along with cellular proteins, functions as a negative-strand replicase producing full-length, negative-sense copies of the genome. Further processing of P123 by nsP2 proteinase activity results in mature non-structural proteins, which together function as a plus-strand replicase yielding both full-length (49S), positive-sense genome copies and 26S subgenomic RNA encoding the structural proteins in the second ORF. Translation of the latter initially yields a polyprotein containing the capsid, PE2, 6K and E1 proteins. The capsid protein is cleaved in the cytosol and the remaining polyprotein undergoes further processing in the rough endoplasmic reticulum and Golgi apparatus, ultimately yielding mature viral structural proteins which assemble at the cell membrane to form mature virus particles.

produce PE2, 6K and E1. These are transported to the Golgi apparatus for glycosylation and further furin-mediated cleavage releases E2 and E3 from PE2, which are then transported onward to the cell plasma membrane. Mature capsid proteins then associate with genomic RNA at the plasma membrane to form nucleocapsids that then acquire an envelope containing viral glycoproteins as they bud from the cell membrane.

Detailed studies of SPDV and SDV (SAV 1 and SAV 2) have confirmed the presence of typical alphaviral genomic stucture (Weston *et al.*, 1999, 2002; Villoing *et al.*, 2000a; Welsh *et al.*, 2000). Their genomes, excluding the poly A tails, were shown to be 11,919 and 11,900 nucleotides, respectively. Both had a 27 nt 5′ non-translated region. The SAV 1 nsP1, nsP2, nsP3 and nsP4 structural proteins consisted of 562, 859, 571 and 609 amino acids, respectively. The nsP1 of SAV 2 was one amino acid shorter, but the nsP3 differed more markedly with the presence of an 8 aa deletion. It has been speculated (Weston *et al.*, 2002) that variability in nsP3 between SAV 1 and SAV 2 could be an indication of host diversity similar to that reported previously for Venezuelan equine encephalitis virus (Oberste *et al.*, 1996). Further partial sequencing of the nsP3 gene of additional

SAV isolates has revealed this region to be hypervariable, grouping isolates into six distinct clades consistent with those obtained with analysis of partial E2 sequence data (Fringuelli *et al.*, 2008). However, the relevance of these findings to the issue of host diversity in general, and the existence of non-salmonid reservoirs in particular, remains to be resolved. The untranslated junction region in both SAV 1 and SAV 2 is 38 nt. The size of the E2, E3 and 6K structural proteins were the same in both viruses (438, 71 and 62 amino acids, respectively). The capsid and E1 proteins of SAV 2 were each one amino acid bigger than those of SAV 1 (282 and 463 aa, respectively). The 3′ untranslated regions of SAV 1 and SAV 2 were shown to be 91 and 90 nucleotides, respectively. Hodneland *et al.* (2005) subsequently showed that the Norwegian salmonid alphavirus (NSAV; SAV 3) also has a typical alphaviral genome.

Many of the conserved motifs and sequence elements identified in other alphaviruses were also shown to be present in SAV 1, SAV 2 and SAV 3. However, a number of differences were also observed: the E1, E2 and capsid proteins were found to be larger than those in other alphaviruses; the glycosylation site in E1 was shown to be in a unique location, while E3 lacked a glycosylation site; the 3′ non-translated region was shorter than most of the other alphaviruses and lacked any repeat sequence elements; unlike the other alphaviruses, shutdown of host cell metabolism did not occur following infection. This downregulation of translation and transcription in vertebrate cells infected with other alphaviruses has been shown to diminish the cellular antiviral response, favouring viral replication to high titre, development of apoptotic changes and cytopathic effect and more efficient replication (Frolov, 2004). nsP2 has been shown to play a key role in this downregulation, with specific mutations favouring persistence of infection *in vitro*. Failure to induce apoptosis following alphavirus infection has been proposed as a mechanism to explain the prolonged persistence of viral RNA in vertebrate hosts with the potential for subsequent reactivation of viral infection (Griffin and Hardwick, 1997).

PD – The Disease

Clinical signs, gross and histopathology

Clinical signs associated with PD may be sudden inappetence, lethargy, an increased number of faecal casts in the cages, increased mortality and ill-thrift (McVicar, 1987; McLoughlin *et al.*, 2002). Affected fish may appear to be unable to maintain their position in the water column/currents in the cages due to muscle damage, predisposing them to erosion and ulceration of the skin and fins (McLoughlin *et al.*, 2002; Taksdal *et al.*, 2007) (Fig. 6.2). They may also be very sensitive to handling, which can lead to sudden death. On a few sites, fish were observed spitting out pellets of food and this was found to coincide with degenerative lesions in the striated muscle of the oesophagus (Ferguson *et al.*, 1986a,b). Fish with normal or good-condition factors may sometimes be seen swimming in a spiralling or circular motion or motionless at the bottom of the cage (similar to SD signs), but swim away if handled. These fish generally have severe skeletal muscle lesions, which prevent normal swimming behaviour and the term 'sudden death syndrome' (SDS) was coined to described this severe manifestation of SAV 1 infection in Ireland (Rodger *et al.*, 1991; McLoughlin *et al.*, 2002). Apparently healthy fish may also die suddenly due to cardiac and skeletal muscle damage and exhaustion (Rodger *et al.*, 1991). This manifestation of PD appears to be more common in Scotland and Norway, where older grower fish in their second year at sea are most commonly affected.

Gross pathology

The principal gross findings at necropsy in the early stages of a PD outbreak are the absence of food in the gut and the presence of yellow faecal casts. Occasionally, petechial haemorrhages are detected over the surface of the pyloric caeca and surrounding fat (Fig. 6.3), which can also be seen in

infectious pancreatic necrosis virus (IPNV) (Taksdal *et al.*, 2007). In the later stages, non-feeding fish with low condition-factor and negligible body fat may be more common due to the failure of the pancreas to recover, and these fish are more susceptible to parasitic and secondary bacterial infections (Fig. 6.4).



Fig. 6.2. Lethargic Atlantic salmon (*Salmo salar* L.) with PD in marine net pen.

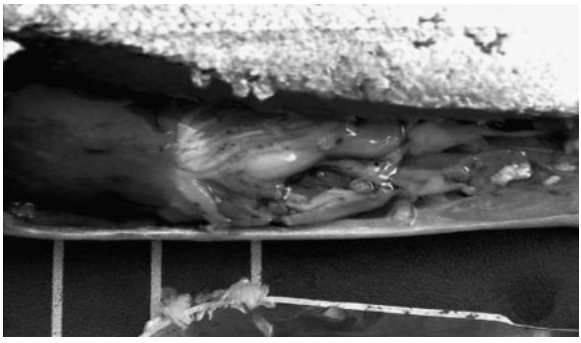


Fig. 6.3. Petechial haemorrhages on caecal fat in acute PD (SAV 1) (Hamish Rodger).

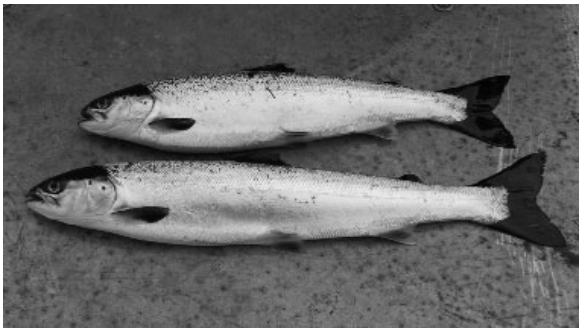


Fig. 6.4. Poor growth post-PD virus infection (SAV 4) (Tom Turnbull).

Histopathology

Pancreas

Pancreatic necrosis was the only histopathological lesion described in the initial description of PD in Scotland (Munro *et al.*, 1984). PD was further investigated by Ferguson *et al.* (1986a), who studied outbreaks on two Scottish marine sites over a 3-month period. This study extended the pathological observations with the first report of cardiomyopathy and skeletal myopathy, including oesophageal muscle lesions, in addition to the pancreatic lesions as described by Munro *et al.* (1984). Ferguson *et al.* (1986a) also found that pancreatic and heart lesions developed concurrently and that mild heart lesions were seen occasionally in apparently healthy fish. This was also observed in sequential studies in Ireland (Murphy *et al.*, 1992; McLoughlin *et al.*, 2002). Ferguson *et al.* (1986a) concluded that the most significant lesion of PD was severe myocardial degeneration.

McVicar (1987) indicated that the heart and muscle lesions described by Ferguson *et al.* (1986a,b) were seen in some of the cases of PD that he investigated. However, he concluded that they were not a consistent finding and were probably due to loss of exocrine pancreas, failure to feed or to the onset of other diseases in weakened fish. In 1994, Rodger *et al.* published a retrospective study of pancreas disease in Scotland. They reviewed the histopathology associated with PD on 19 sites over a 2-year period (1991–1993) and found that 78% of fish from these sites had cardiac and/or skeletal myopathies associated with PD. They considered myopathies to be very significant in the pathogenesis of PD outbreaks.

In 1989, Poppe *et al.* reported that PD had been a sporadic problem in farmed Atlantic salmon in Norway. The timing of the outbreaks, clinical signs and gross pathology of what these authors referred to as PD were similar to the earlier descriptions of Munro *et al.* (1984) and Ferguson *et al.* (1986a,b). However, the histological lesions described in the pancreas and gut resembled more closely those associated with IPNV (McKnight and Roberts, 1976), which was very prevalent in Norway at that time (Krogsrud *et al.*, 1989). The heart lesions described were similar to those recorded by Ferguson *et al.* (1990) in cardiomyopathy syndrome (CMS) in older Atlantic salmon growers in Norway, where these authors speculated that CMS might be a severe manifestation of PD infection. The observed heart lesions were more severe than those described previously in PD outbreaks in Scotland (Ferguson *et al.*, 1986a,b) and later in Ireland (Murphy *et al.*, 1992).

In 1992, Murphy *et al.* published the first sequential study of naturally occurring PD. They observed a 1-week time lag between the occurrence of acute histological lesions and subsequent clinical signs of inappetence and lethargy. In general, Murphy *et al.* (1992) found that clinical signs of PD were very non-specific on these sites and required considerable experience to identify at an early stage. A similar sequential study and a review of 40 diagnostic cases (from 1991 to 1994) of naturally occurring PD in Ireland by McLoughlin *et al.* (2002) produced the first complete description of the clinical signs, gross pathology and the range and distribution of histological lesions associated with PD. The results of this sequential study including sequential pancreatic pathology were very similar to those described by Murphy *et al.* (1992). Despite regular sampling of the populations in both of these studies (Murphy *et al.*, 1992, and McLoughlin *et al.*, 2002) and a recent study in Norway by Taksdal *et al.* (2007), relatively small numbers of fish were observed with acute pancreatic lesions. Murphy *et al.* (1992) found less than 2% of fish with acute pancreatic lesions, McLoughlin *et al.* (2002) detected 4% and Taksdal *et al.* (2007) reported 5%. This finding suggests that it is very difficult to detect early acute stage PD and this may be one of the reasons it is difficult to isolate the infectious agent from field material. The acute phase is relatively short-lived, with rapid destruction of the majority of pancreatic acinar tissue (McLoughlin *et al.*, 1996) and a variable inflammatory response ranging from no inflammation to moderate

mononuclear cell infiltration and/or fibrosis of the periacinar tissue. Chronic pancreatic lesions were classified as significant loss of pancreatic acinar tissue with or without fibroplasia of periacinar tissue (Fig. 6.5). In recovering fish, regeneration of exocrine tissues appeared to take place as early as 4 weeks after infection (Munro *et al.*, 1984; Murphy *et al.*, 1992; McLoughlin *et al.*, 2002), although it has been reported that the chronic pancreatic lesions might be more prevalent and persist for a longer period in Norwegian outbreaks of PD (Taksdal *et al.*, 2007). Fish with extensive fibrosis of the periacinar tissue tended to become runts.

Heart

On a temporal basis, acute heart lesions were observed concurrently or slightly lagging behind the acute pancreatic acinar necrosis. Lesions included multifocal cardiomyocytic necrosis, with affected cells having a shrunken, deeply eosinophilic cytoplasm and pyknotic nuclei. Both compact and spongy ventricular and atrial muscles were affected to varying degrees, ranging from mild focal lesions to severe diffuse involvement of the entire heart musculature. Hypertrophy of myocardial myocytic nuclei, particularly at the junction of the spongy and compact ventricular muscle, was evident in the recovery phase (Ferguson *et al.*, 1986a; McLoughlin *et al.*, 2002) (Fig. 6.6). Mitotic figures were a consistent feature in affected hearts in smolts, but were seen less frequently or were absent in older affected fish (M.F. McLoughlin, unpublished observations, 2008). The ability of younger fish to replace damaged cardiomyocytes by cell division, which is not present in older fish, may result in a different

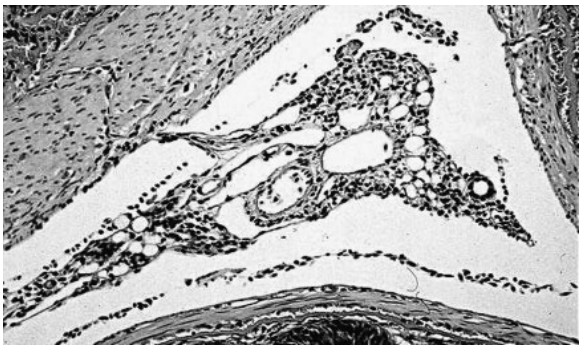


Fig. 6.5. Total loss of exocrine pancreatic acinar cells in chronic PD (SAV 1) in farmed Atlantic salmon.

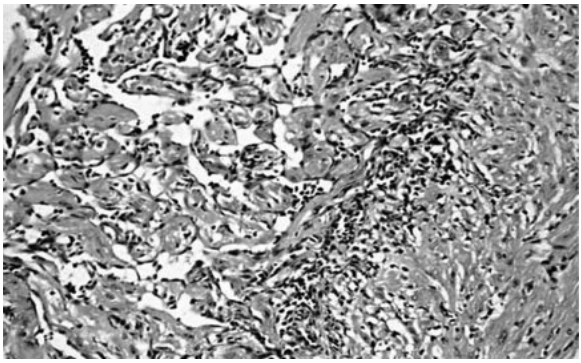


Fig. 6.6. Inflammation of junction between compact and spongy heart muscle in chronic PD (SAV 1) in farmed Atlantic salmon.

pathogenesis and impact of PD infection in the hearts of some Atlantic salmon if infected at different stages (smolt versus grower) of marine production.

Skeletal muscle

Skeletal muscle lesions first appear 3–4 weeks after the appearance of pancreatic and heart lesions; therefore, fish sampled in late phase disease may have only skeletal muscle lesions. The muscle lesions consist of hyaline degeneration, with swollen fragmented eosinophilic sarcoplasm, central migration of myocytic nuclei and subsequent invasion of the sarcoplasm by phagocytic macrophages. Subsequent fibrosis of the surrounding tissues may or may not be present, depending on the severity of the primary muscle damage (Fig. 6.7). Recovering cells tend to have a basophilic appearance, which differentiates them from undamaged cells. The red muscle fibres show similar sarcoplasmic changes, but in many fish, a higher proportion of the red muscle compared to white muscle fibres tends to be damaged (Fig. 6.8). These aerobic red muscles are used mostly when the fish is swimming slowly at constant speed, while the anaerobic white muscle fibres that make up the bulk of the fish musculature are used mostly when the fish has to swim rapidly. Recovering red muscle fibres tend to be very basophilic, with variable inflammation and fibrosis in the surrounding tissues. In severe cases, the entire cross-section of lateral line muscle

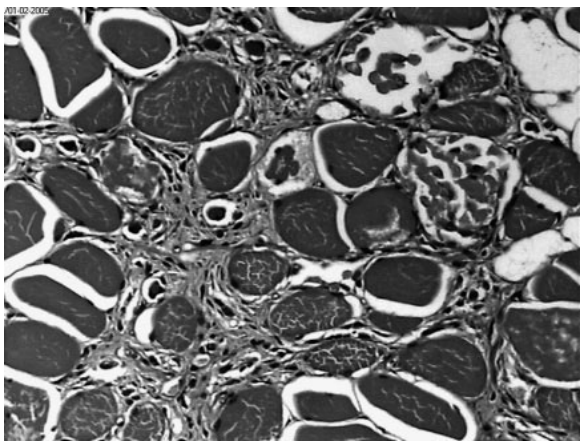


Fig. 6.7. White (anaerobic) muscle fibre degeneration and fibrosis in chronic PD in experimental (SAV 3) in Atlantic salmon.

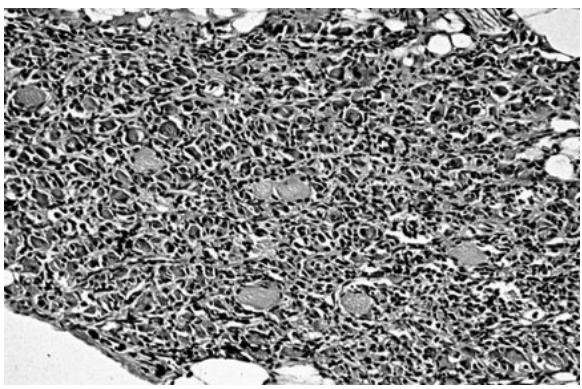


Fig. 6.8. Diffuse degeneration and inflammation of the aerobic red muscle fibres in chronic PD (SAV 1) in farmed Atlantic salmon.

may be affected. These muscle lesions explain the sluggish swimming behaviour of affected fish and contribute significantly to PD-related mortalities (Rodger *et al.*, 1991).

Other organs

Kidney lesions have been described in late-stage cases of PD in Norway (Taksdal *et al.*, 2007). Affected kidneys contain numerous interstitial cells, which are filled with eosinophilic material. It is not clear if these are eosinophilic granular cells or cells containing protein breakdown products (Taksdal *et al.*, 2007). This type of kidney lesion is seen rarely elsewhere, although it has been seen in cases from the Shetland Islands, Scotland (McLoughlin and Graham, 2007). Focal gliosis in the brain is a rather non-specific nervous tissue lesion but it is being associated increasingly with SAV infections in fish and has been recorded recently for the first time in experimental SAV 1 infection (McLoughlin *et al.*, 2006).

These sequential studies confirmed that PD affected a number of key organs in a definite temporal and consistent manner. The key lesions in naturally occurring PD appear in the pancreas, heart and skeletal muscle of PD-affected fish. The severity and distribution of the observed lesions depend on the timing of the sample in relation to the onset of PD in fish in cages. If fish were sampled in the early stages of the infection, the skeletal muscle lesions might be very mild, if present at all, and could be overlooked easily. Conversely, if fish were examined in mid- to late-stage infection, many of them would have pancreatic and cardiac tissue in the recovery phase and the fish with the most severely damaged muscles would probably have died (Fig. 6.9).

Sleeping Disease

Clinical signs, gross and histopathology

Sleeping disease (SD) was first observed in France (Boucher *et al.*, 1994) and affected rainbow trout at all stages of production. The characteristic clinical presentation is of affected fish lying on their side on the bottom of the tank, hence the name 'sleeping' disease (Fig. 6.9). This sign is due primarily to extensive necrosis of skeletal red muscle. This chronic lesion follows sequential histological lesions of the exocrine pancreas and heart, similar to those described earlier for PD (Boucher and Baudin Laurencin, 1996). Reported mortality levels have been very variable, from negligible to over 22% in affected populations (Boucher and Baudin Laurencin, 1994; Graham *et al.*, 2003a).

The first comparative study of the chronological development of histological lesions, viraemia and neutralizing antibody responses associated with a naturally occurring outbreak of sleeping disease in rainbow



Fig. 6.9. Rainbow trout (*Oncorhynchus mykiss* (Walbaum)) showing typical 'sleeping' behaviour, resulting from SAV 2 infection; some have exophthalmos (Jeanette Castric).

trout was carried out recently by Graham *et al.* (2007b). Viraemia precedes both the onset of histological changes and clinical signs, and virus neutralizing (VN) antibodies persist and may be used to detect infection retrospectively for some time afterwards. This is similar to the disease development in naturally occurring PD outbreaks (McLoughlin *et al.*, 2002; Graham *et al.*, 2005). The development of histopathological lesions also reflected the pattern seen in experimental infections with both SAV 1 and SAV 2 (Boucher and Baudin Laurencin, 1996; McLoughlin *et al.*, 1996; Kerbart Boscher *et al.*, 2006) and noted previously by Boucher and Baudin Laurencin (1994) in naturally occurring SD in France.

Differential Diagnosis

The other conditions in salmonids which need to be taken into account in a differential diagnosis of salmonid alphavirus diseases are IPN (Roberts and Pearson, 2005), cardiomyopathy syndrome (CMS) (Ferguson *et al.*, 1990), heart and skeletal muscle inflammation (HSMI) (Kongtorp *et al.*, 2004a) and nutritional myopathies. CMS has been reported in Norway and northern Scotland only and not in Ireland (Ferguson *et al.*, 1990; Rodger and Turnbull, 2000). HSMI has been described in the region immediately north of the PD endemic zone in Norway and in one isolated suspect case in Scotland (Kongtorp *et al.*, 2004a; Ferguson *et al.*, 2005). Currently, the aetiology of only IPN, PD and SD are known. It has been speculated for some time that both HSMI and CMS are caused by viruses other than SAV, with electron microscopic findings of viral-like particles associated with CMS and HSMI lesions (Grotmol *et al.*, 1997; Karlsen *et al.*, 2005; Watanabe *et al.*, 2006) and experimental reproduction of HSMI using tissue homogenates from naturally affected fish (Kongtorp *et al.*, 2004b). However, evidence of SAV has also been found in cases of CMS and haemorrhagic smolt syndrome in Norway (Nylund *et al.*, 2003; Hodneland *et al.*, 2005). Further studies are required to elucidate the cause of these two conditions. The main histological difference between PD and these CMS and HSMI conditions is the absence of pancreatic lesions in HSMI and CMS and the presence of liver pathology consistent with chronic heart failure in CMS, with the most significant lesions being described in the heart. HSMI was described originally in salmon 5–9 months post-transfer to the sea in Norway, while CMS was diagnosed more typically in fish during their second year in sea cages. IPN can be differentiated from PD and SD on both histological and immunohistochemical grounds (Evensen and Rimstad, 1990; McLoughlin, 1997; McLoughlin *et al.*, 2002; Taksdal *et al.*, 2007).

Diagnostic Methods

A preliminary diagnosis of PD and SD can be based on clinical signs and histopathology, with virus isolation, serology and RT-PCR techniques available for confirmation. Summaries of the diagnostic panel for SAV diseases and similar pathologies are presented in Tables 6.2 and 6.3.

Viral culture

Although long suspected of having a viral aetiology, primary isolation of the viruses responsible for PD and SD took many years to achieve. As a consequence, much of the early work on pathogenesis was conducted using extracts from tissue homogenates (Boucher *et al.*, 1994, 1995; Houghton, 1995).

The first successful isolation of SAV from Atlantic salmon was made by co-cultivation of Chinook salmon embryo (CHSE-214) cells with kidney from affected fish (Nelson *et al.*, 1995). Following serial passage, viral growth was revealed by the emergence of a cytopathic effect (CPE) that was absent on the original culture. The CPE initially consisted of irregular small foci of pyknotic vacuolated cells (Fig. 6.10).

The speed with which CPE developed, and the extent of the cell monolayer involved,

Table 6.2. Diagnostic panel for PD and related pathologies based on experimental infections with SAV at 12–14°C.

Test	Preacute	Acute	Subacute	Chronic	Recovered	CMS	HSMI
DPI[a]	0–7	7–14	14–21	21–42+	>42	?	>42
PD signs		↓Appetite	Casts	Morts	Runts	Sudden death	
Virus	Serum, heart	Serum, heart	Serum, heart	–	–	–	–
PD histo-lesions	NA	Pancreas, heart	Pancreas, heart	Heart, muscle, pancreas	Heart, muscle, pancreas	Heart	Heart, muscle
IHC	NA	+	+	+	+/–	–	–
SAV RT-PCR (heart)	+	+	+	+	+	–	–
Serology	–	–	–	+	+	–	–

[a]Days post-infection. NA, not applicable.

Table 6.3. Diagnostic panel for SD (SAV 2).

Test	Preacute	Acute	Subacute	Chronic	Recovered
DPI[a]	4–13	14–21	22–35	36–49	>49
SD signs	Low mortality	Low mortality	↓Appetite	Rising mortality, exopthalamus	Lethargy, sleeping, mortality
Virus	Serum, heart, kidney, brain	Serum, heart, kidney, brain	–	–	–
PD histolesions	+Pancreas	+Pancreas	+Pancreas, heart	+Heart, muscle, pancreas	+Heart, muscle, pancreas
IHC	+Pancreas	+Muscle	ND	ND	ND
RT-PCR	+	+	+	+	+
Serology	–	+	+	+	+

[a]This table is based on experimental infections at 10 ± 1°C. ND, not done.

increased with serial passage. Similar findings were reported for the first isolation of SAV 2, which produced a CPE after serial passage of kidney homogenate in both CHSE-214 and rainbow trout gonad (RTG-2) cells (Castric *et al.*, 1997). Re-isolation of these cultured viruses from experimentally infected fish was found to be much more straightforward than the original isolation processes, with an extensive CPE evident on the first passage. Both the development of CPE with serial passage and the relative ease of re-isolation of cultured virus following fish passage suggest a degree of selection or adaptation that may reflect the quasispecies nature of RNA viruses (Domingo *et al.*, 2006). Karlsen *et al.* (2005) recently have suggested a molecular basis for the emergence of CPE on serial passage, which first appeared on the 13th passage in CHSE-214 cells coincident with a change from serine to proline at amino acid 206 of E2.

Following the initial successes at viral isolation, SAV was isolated successfully from a range of other tissues, including heart and brain, following similar protocols (Christie *et al.*, 1998; Rowley *et al.*, 1998; Graham *et al.*, 2003a). Nevertheless, the making of isolates from tissues from field material based on the observation of CPE on

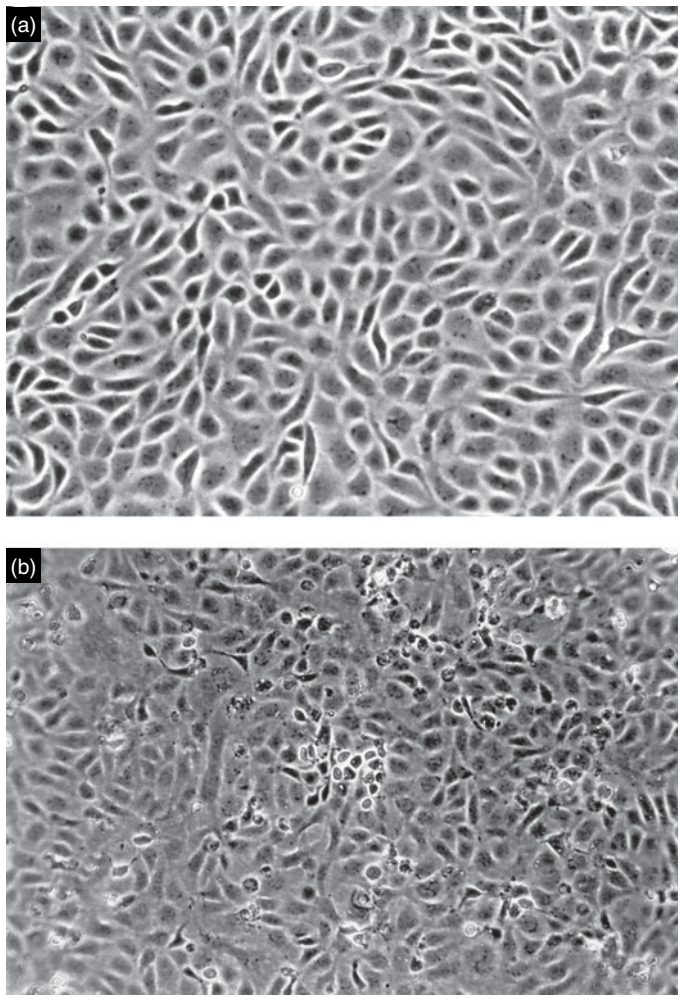


Fig. 6.10. Early SAV-induced cytopathic effect (CPE) in CHSE-214 cells (bottom) with uninfected cells (top) for comparison.

serial passage continued to be labour-intensive and yielded relatively small numbers of isolates. A number of factors are considered to have contributed to this. An early transmission study by Houghton (1995) showed that the presence of infectious virus largely disappeared at the same time as the first pathology, affecting the pancreas, was observable. More recently, Kerbart Boscher *et al.* (2006) compared the viral titres in serum, kidney and brain at several time points following i.p. inoculation of rainbow trout with SAV 2. Virus was recovered from all three sample types up to 21 days post-infection (d.p.i.), with mean viral titres being highest in serum, followed by kidney and then brain. However, clinical signs of sleeping disease only appeared in this study after 35 d.p.i., when virus was no longer detectable. Similarly, in a prospective longitudinal study of a field outbreak of SD, Graham *et al.* (2007b) reported that the detection of viraemia preceded the appearance of clinical signs by 2 weeks. Taken together, these studies highlight the need to collect samples from fish in the early stages of

infection. Tissue tropism and pathogenesis studies suggest that virus or viral RNA is present in a wide range of tissues, including heart, gill, pseudobranch, kidney, pancreas and somatic muscle, indicating that these are suitable for viral isolation (Houghton, 1995; Kerbart Boscher *et al.*, 2006; Andersen *et al.*, 2007). In practice, selection of tissues for viral isolation requires an awareness of the clinical signs, with collection of samples as soon as PD or SD is suspected, and the inclusion of apparently healthy as well as diseased fish.

A reliance on CPE as an indicator of SAV growth is also likely to hinder successful isolation of the virus, given that viral growth can be non-cytopathic for a number of passages, and that CPE, once present, can be both relatively nondescript and variable in extent (Nelson *et al.*, 1995; Castric *et al.*, 1997; Desvignes *et al.*, 2002; Graham *et al.*, 2003a; Karlsen *et al.*, 2005). One approach that has been used to demonstrate the titre of cultured viruses more reliably is the phenomenon of heterologous interference between SAV and a secondary cytopathic virus such as viral haemorrhagic septicaemia virus (Desvignes *et al.*, 2002; Villoing *et al.*, 2000a). However, this technique has been largely superseded by the introduction of immunostaining techniques using virus-specific MAb, which allows the demonstration of replicating virus even in first passage material in the absence of CPE (Rowley *et al.*, 1998; Todd *et al.*, 2001; Graham *et al.*, 2003a; Jewhurst *et al.*, 2004; Kerbart Boscher *et al.*, 2006).

Although it has been recognized since the early stages of SAV research that primary infection was followed by a viraemia (Houghton, 1995; Desvignes *et al.*, 2002; Kerbart Boscher *et al.*, 2006), serum was not used typically for isolating the virus. Jewhurst *et al.* (2004) have shown the relative ease with which isolates can be obtained from serum, with positive results as early as 3 days post-inoculation when combined with immunostaining. While less sensitive than serial passage in cell culture, this approach has a number of benefits, including the need for minimal sample preparation of tissues, the ability to screen large numbers in a microtitre format and the possibility of combining it with serological screening for regular monitoring of fish populations (Graham *et al.*, 2007a,b). Using this approach, over 200 viraemic salmon and trout sera from field submissions have now been identified at the Veterinary Sciences Division [VSD], Northern Ireland (D.A. Graham, unpublished data, 2008).

A number of other factors can influence the success of viral isolation and propagation. Lopez-Doriga *et al.* (2001) showed that the titre obtained with a salmon isolate grown in the presence of fetal bovine serum (FBS) was between 10- and 100-fold higher than that achieved without FBS. Cell line and temperature of incubation may also have an effect. The first isolations of SAV 1 (SPDV) and SAV 2 (SDV) were both made in CHSE-214 at 15 and 14°C, respectively (Nelson *et al.*, 1995; Castric *et al.*, 1997). While CHSE-214 continues to be used routinely for processing of samples and viruses from Atlantic salmon, RTG-2 is used typically for rainbow trout. Initial studies on cell line susceptibility used the presence of CPE as an indicator of viral replication. On this basis, it was reported that a rainbow trout fibroblast cell line was permissive, while fathead minnow (FHM), bluegill fry (BF-2), Atlantic salmon fibroblast (AS-6) and *epithelioma papulosum cyprini* (EPC) cells were non-permissive (Nelson *et al.*, 1995). However, recent immunostaining studies have shown that SAV will grow in a range of additional cell lines, including BF-2, FHM, salmon head kidney 1 (SHK-1) and TO (Graham *et al.*, 2008). Of the additional cell lines, TO and BF-2 were shown to perform best, in terms of both ability to make primary isolates from Atlantic salmon and rainbow trout and the viral titres obtained, which were significantly higher than those observed in the other cell lines, including CHSE-214 and RTG-2. Moriette *et al.* (2006) also reported that BF-2 cells supported replication of SDV to high titre. It is noteworthy that the non-salmonid alphaviruses are considered to be cytopathic in vertebrate cells but non-cytopathic in insect cells (Frolov, 2004). This is considered to reflect their epidemiology, with a requirement for a high virus titre in vertebrates to enable transmission by arthropods, whereas non-lytic

infection in arthropods is consistent with chronicity or persistence of infection.

Consistent with the initial reports on isolation, different laboratories continue to use temperatures in the range 10–15°C for isolation and propagation of SAV (Graham *et al.*, 2003a; Hodneland *et al.*, 2005; Kerbart Boscher *et al.*, 2006). There is evidence that the optimal temperature for growth may vary with viral strains and cell lines. Villoing *et al.* (2000a) and Moriette *et al.* (2006) reported that trout isolates (SAV 2) in CHSE-214 and RTG-2 grew better at 10°C. Moriette *et al.* (2006) also reported an association between virulence and growth at a higher temperature. Lopez-Doriga *et al.* (2001) found that a Scottish Atlantic salmon isolate grew to similar titres in CHSE-214 at 10°C, 15°C and 20°C. Graham *et al.* (2008) found that an Irish Atlantic salmon isolate grew best at 15°C in TO and RTG-2 cells, but poorly or not at all at 4°C, 10°C or 20°C, while growth in CHSE-214 was similar at both 10 and 15°C. This relatively narrow permissive temperature range for SAV is in contrast to the wide range reported for other alphaviruses that is considered necessary to allow their replication in both arthropod and mammalian or avian vertebrate hosts (Strauss and Strauss, 1994).

SAV is stable when frozen and stored at –20°C or below, but the half-life of the virus decreases as the temperature increases from 4°C (Graham *et al.*, 2007d). Therefore, samples for viral isolation should be shipped to the laboratory on ice or frozen to prevent reduction or loss of infectivity during transport.

Immunodiagnosis and serology

The development of effective immunohistochemical (IHC) techniques for the detection of SAV antigens has proven difficult. Taksdal *et al.* (2007) were able to detect SAV in acute pancreatic lesions in a minority of fish from a few outbreaks. Heart, skeletal muscle and kidney in the same study were consistently negative using immunohistochemistry. Since acute pancreatic lesions are observed in only a minority of affected fish (McLoughlin *et al.*, 2002), the diagnostic potential of this technique is limited. More recently, Moriette *et al.* (2005) have used an anti-E1 MAb to demonstrate viral antigen in red muscle and pancreas. An SAV antigen capture ELISA based on a pair of MAbs has also been described (Todd *et al.*, 2001), but is not used routinely.

A traditional reliance on viral isolation techniques, coupled with profound differences between the humoral immune responses of mammals and fish, has resulted in serological assays being little used in aquaculture diagnostics (Denzin and Staak, 2000). Thus, relative to mammals, fish produce a more limited antibody repertoire (Du Pasquier, 1982; Wilson and Warr, 1992) and their antibody response is influenced by temperature and limited to a single isotype (analogous to IgM), which does not show the isotype switching, maturation of affinity or true anamnestic response characteristic of mammals (Corbel, 1975; Wilson and Warr, 1992). Nevertheless, a test capable of detecting viral neutralizing (VN) antibodies to SAV 1 was developed quickly following first isolation of SPDV (McLoughlin *et al.*, 1996). Studies on sera from experimental infections demonstrated that VN antibodies could be detected as early as 10 d.p.i., with all fish having seroconverted by 21 d.p.i. Comparable findings for SAV 1, SAV 2 and SAV 3 have also been reported by others using VN testing, with the disappearance of viraemia typically coinciding with the development of a detectable VN response (McLoughlin *et al.*, 1996; Lopez-Doriga *et al.*, 2001; Desvignes *et al.*, 2002; Kerbart Boscher *et al.*, 2006; Christie *et al.*, 2007). Published methods have varied in a number of ways, including the strain and amount of virus used, incubation times, volumes, serum dilutions, use of complement and interpretation, making direct comparison of results difficult. There is a recognized need for cross-evaluation and further validation of these tests, particularly in relation to the cross-reactivity between different SAV subtypes. The first VN test to be described (McLoughlin *et al.*, 1996) was carried out in a 24-well format and read for the presence

or absence of viral CPE after 7 days. This assay was limited in throughput and also by the level of expertise required to read it accurately. It has subsequently been modified extensively to overcome these limitations. The current method uses a 96-well microtitre format which can be read easily after 3 days due to the incorporation of an MAb-based immunostaining procedure to demonstrate viral growth (Graham *et al.*, 2003b). The inclusion of an immunostaining step allows sera to be screened in parallel for the presence of viraemia (Jewhurst *et al.*, 2004). This combined screening for virus and antibody has been found to be a useful diagnostic tool at a population level, having the potential to allow early detection of infection and implementation of husbandry procedures to minimize stresses on the fish and is now being used in some areas for regular on-farm surveillance.

In addition to straightforward diagnostic applications, VN assays have now been used to demonstrate the protective effect of the humoral antibody response and in pathogenesis and prevalence studies (McLoughlin *et al.*, 1998; Desvignes *et al.*, 2002; Graham *et al.*, 2003b, 2005; Graham, 2005; Kerbart Boscher *et al.*, 2006; Christie *et al.*, 2007).

Following clinical outbreaks of disease, seroprevalence can reach 100% of sampled fish rapidly (Graham *et al.*, 2005). In contrast to viraemia, antibodies can be detected in sera for extended periods following infection. In longitudinal studies of clinical outbreaks, up to 90% of samples were still found seropositive at the end of the study, 36 weeks after initial seroconversion, although the geometric mean titres tended to wane over this time (Graham *et al.*, 2005). Titres obtained from field sera ranged from 1/20 (the lowest dilution tested) to ≥1/1280. Thus, the persistence of serum VN antibodies makes them a useful tool for screening populations to determine previous exposure to SAV (McLoughlin *et al.*, 1998; Graham *et al.*, 2003b). A longitudinal serological and virological study has demonstrated for the first time that subclinical infection is also possible (Graham *et al.*, 2007a). In this case, infection spread slowly, with a low prevalence of viraemic fish detectable over an extended period, and consequently the seroprevalence increased much more slowly.

The use of serum for serological and virological screening also offers the possibility of non-lethal sampling. While VN testing is the recognized 'gold standard' for serological testing, some sera can be cytotoxic, particularly at low dilution (Graham *et al.*, 2003b; Kerbart Boscher *et al.*, 2006). To maintain the integrity of samples, and minimize the frequency of cytotoxicity, serum should be separated from blood cells and shipped to the laboratory at 4°C. It is possible that the development of other serological tests, such as ELISA, will overcome this problem in due course, although none are currently in routine use.

Molecular probes/techniques

Several conventional SAV RT-PCR tests targeting sequences from a range of viral genes including E1, E2, nsP3 and nsP4 have been described (Villoing *et al.*, 2000b; Hodneland *et al.*, 2005; Weston *et al.*, 2005; Graham *et al.*, 2006; Hodneland and Endresen, 2006). Villoing *et al.* (2000b) were able to detect viral RNA in homogenized juvenile rainbow trout fry up to 70 d.p.i. using a primer pair amplifying a 284 bp product of the E2 gene. In contrast, no virus could be cultured after 41 d.p.i., demonstrating the greater analytical sensitivity of the RT-PCR method.

More recently, real-time RT-PCR protocols based on SYBR green (Graham *et al.*, 2006) or Taqman (Hodneland and Endresen, 2006; Christie *et al.*, 2007) detection chemistries have been described. Hodneland and Endresen (2006) developed three separate assays, including a screening assay designed to detect SAV subtypes 1, 2 and 3, one to detect only SAV 3 strains and one to detect only SAV 1 strains. These Taqman assays were shown to be specific and highly sensitive, with limits of detection in the range 0.01–0.08 $TCID_{50}$. When compared with a conventional RT-PCR, the screening real-time assay was found to be 10- to 100-fold more sensitive. When applied to field

samples, this greater sensitivity resulted in positive results using real-time RT-PCR for some samples found to be negative using the conventional assay. One of the aims of this work was to develop assays that could be used for initial screening and subsequent strain typing of isolates. The identification of additional subtypes after this work was published means that further validation is required. While it has been shown that the screening assay will detect all six currently described subtypes, the assay designed to be SAV 1-specific also detects additional subtypes (D. Graham and E. Fringuelli, unpublished data). The SYBR green-based protocol described by Graham *et al.* (2006) was also specific and sensitive, with a detection limit of 1.5 $TCID_{50}$/reaction. Based on the large number of sequences used in the design of the primers, and subsequent testing, this protocol is considered capable of detecting a wide range of isolates. It was found to be much more sensitive than the 3-day virus isolation test as applied to serum (Graham *et al.*, 2007a).

Villoing *et al.* (2000b) found that, when testing samples from field outbreaks of SD, more reliable results were obtained with kidney and pyloric caeca than from heart or brain. Using the real-time protocol described by Christie *et al.* (2007), it was possible to detect viral RNA in heart samples for at least 140 d.p.i., whereas serum was rarely positive later than 21 d.p.i. A tropism study by Andersen *et al.* (2007) based on experimental infections found that RT-PCR signals persisted longest in pseudobranch and heart. Similar field studies from outbreaks of PD have shown that RT-PCR signals are most persistent in gill and heart, followed by pancreas, kidney, brain and then serum (D. Graham and E. Fringuelli, unpublished data). Thus, positive serum results appear to indicate current, active infection, whereas positive tissue results, particularly for heart, gill or pseudobranch, may indicate historical exposure rather than active infection at the time of sampling. Further studies are required to determine whether persistent RNA signals indicate the presence of a carrier state from which viable virus can be recovered (Levine and Griffin, 1992; Griffin and Hardwick, 1997).

Pathogenesis of PD and SD

The earliest attempts at experimental reproduction of PD were carried out at the Marine Laboratory in Aberdeen using kidney homogenates from affected fish (McVicar, 1987, 1990; Raynard and Houghton, 1993). A limitation of this work was that only pancreatic pathology was monitored, but these studies showed that cohabitant fish became diseased, as determined by pancreas pathology, that the pathogenicity was not diminished by subsequent passage of kidney material from experimentally infected fish and that the number of diseased fish was unaltered by passage of kidney homogenates through a 0.22 μm filter. All of these findings strongly suggested a viral aetiology.

Similar experimental studies carried out by McLoughlin *et al.* (1995) and Murphy *et al.* (1995) in salmon parr in fresh water confirmed the infectious nature of infected tissue homogenates, and both studies extended the observations to include the first descriptions of experimentally induced cardiomyopathy, which developed concurrently with pancreatic lesions.

Subsequently, Houghton (1995) found that plasma, blood leucocytes, splenocytes and kidney homogenates were all infective following i.p. injection of Atlantic salmon post-smolts with PD-affected kidney homogenates. She showed that the kinetics of infectivity was temperature dependent, with a more rapid dissemination of infection at 14°C compared with 9 and 6°C, and that infectivity was relatively short-lived. Murphy *et al.* (1995) reported that tissue homogenates from PD-affected fish were inactivated by chloroform and suggested PD was caused by a virus with a lipid envelope. Comparative experimental transmissions of PD in Atlantic salmon, rainbow trout and brown trout in fresh water were also carried out. Using pancreas pathology as the sole diagnostic criterion, these experiments showed that, whereas Atlantic

salmon developed total loss of pancreatic acinar cells, brown trout showed minimal pancreatic changes and rainbow trout had lesions of intermediate severity (Boucher *et al.*, 1995). In experimental infections of Atlantic salmon and rainbow trout with SAV 1 and SAV 2, Weston *et al.* (2002) confirmed that the disease lesions in pancreas, heart and muscle induced by both viruses were very similar in both species of fish. They observed that, while infection with SAV 1 and SAV 2 produced similar levels of histopathological lesions in rainbow trout, SAV 2 induced significantly less severe lesions in salmon than did SAV 1 (Tables 6.2 and 6.3).

McLoughlin *et al.* (1996) were the first to show conclusively that the newly isolated 'toga-like' virus from PD-affected Atlantic salmon post-smolts (Nelson *et al.*, 1995) could reproduce concurrent lesions in the pancreas and heart, and subsequently in skeletal muscle in post-smolts, indistinguishable from those described in naturally occurring PD (Murphy *et al.*, 1992; McLoughlin *et al.*, 2002). SAV 1 virus was re-isolated from inoculated smolts at 7, 10, 15 and 21 d.p.i. and from in-contact fish at 14 and 21 d.p.i. Neutralizing antibody was first detected at 10 d.p.i. and in the cohabitant fish 11 days later, suggesting an incubation period of 7–10 days in seawater at 12–15°C. French workers have reported similar results, with slight temporal variations, in Atlantic salmon parr (Desvignes *et al.*, 2002).

Recent experimental SAV 1 studies have examined the effects of fish strain on the outcome of SAV 1 infection (McLoughlin *et al.*, 2006). Highly significant differences in the severity of lesions in the pancreas at 21 d.p.i. were detected, and there were also significant differences in the prevalence and severity of lesions in heart and skeletal muscle at 21 and 35 d.p.i., respectively. Differences between the number of antibody-positive fish in each challenge group were found at 28 and 35 d.p.i. ($p<0.1$). Highly significant differences ($p<0.01$) in the geometric mean titres of seropositive fish were detected at day 28. These results using a challenge model demonstrate that there are strain differences in the outcome to experimental SAV 1 infection in commercial farmed Atlantic salmon (McLoughlin *et al.*, 2006).

Experimental studies have also been used to compare the pathogenicity of a Norwegian SAV 3 isolate with an Irish SAV 1 isolate in Atlantic salmon parr (Christie *et al.*, 2007). Sequential samples of tissue and blood were collected during a period of 20 weeks post-infection and subjected to viral isolations from kidney tissue and serum. Viral nucleic acid was detected in heart tissue and serum using real-time RT-PCR test, with detection of specific antibodies using viral neutralization assay and histopathological examination.

Successful reproduction of pancreas disease was obtained by i.p. injection with both isolates. The prevalence and severity of lesions in the pancreas, heart, skeletal muscle and brain were similar in both groups, with only subtle differences. No mortality was observed post-infection in either group. For the first time, a significant reduction in weight gain was noted in fish experimentally infected with SAV 1 and SAV 3 when compared to uninfected control fish. Re-isolation of virus from kidney tissue was performed at 7 and 14 d.p.i. only and was positive for both test groups at both sampling points. Isolation of virus from sera from both groups was positive 4–14 d.p.i., but was negative at later sampling points when antibody production had started. Virus could only be isolated during the acute phase of the disease. Specific neutralizing antibodies could be detected from 21 d.p.i. for both test groups and until the end of the experiment at 140 d.p.i. Peak antibody titres were seen 70 d.p.i. Using real-time RT-PCR, SAV 1-specific RNA was detected frequently up to 14 d.p.i. in serum, and occasionally thereafter. In contrast, viral RNA could still be detected in the heart tissue of fish from both groups for at least 140 d.p.i.

A similar study has been reported recently by Andersen *et al.* (2007), where real-time RT-PCR techniques were applied to a range of tissues (pseudobranch, gill, atrium, ventricle, kidney, pancreas, muscle) from Atlantic salmon smolts that had been

inoculated intraperitoneally with SAV 1 or SAV 3 isolates. Their results indicated that the pseudobranch and heart tissue (ventricle) were the most useful tissues for real-time RT-PCR assays, regardless of disease status, detecting previous SAV infection for up to 6 months post-infection. The pancreas was the least useful tissue, probably due to the rapid destruction of this tissue during the viraemic phase.

French workers have completed a detailed experimental SD infection in 1-year-old rainbow trout. This study reproduced all the pathognomonic features of naturally occurring SD, including typical sequential pancreatic, heart and skeletal muscle lesions, with neutralizing antibodies to SDV from 14 to 70 d.p.i. Cumulative mortality reached 18.7% in 44 days (Kerbart Boscher *et al.*, 2006).

Immunity and Vaccination

Very few studies have been carried out on the specific immune responses to SAV infections in fish. Houghton (1994) provided evidence that Atlantic salmon that had recovered from infection with tissue homogenates as parr developed a very strong resistance to reinfection, which was maintained for at least 9 months. Further insight into the pathogenicity and immune response was reported by Houghton (1995), who demonstrated that plasma, blood leucocytes and lymphoid tissue were all involved in the dissemination of PD infection throughout the fish. The nature of the immune response is not understood fully. Houghton and Ellis (1996) and Murphy *et al.* (1995) demonstrated that passive immunization of Atlantic salmon parr and smolts with convalescent PD antisera from naturally and experimentally infected fish gave significant protection following challenge with PD-infected homogenates. Boucher and Baudin Laurencin (1996) also demonstrated cross-protection with SAV-infected homogenates from Atlantic salmon and rainbow trout as primary and secondary challenge inocula in both Atlantic salmon and rainbow trout.

Desvignes *et al.* (2002) carried out SAV 1 challenge experiments in Atlantic salmon parr at 14°C and observed similar histopathological lesions to those described previously in SAV experimental infections. In addition, they also showed rapid haematogenous spread and the absence of secondary viraemia. No interferon was detected, but this might have been due to the lack of sensitivity in the technique used. The phagocytic activity of head kidney leucocytes was always significantly higher in the infected fish than in the control fish. Lysozyme and complement levels were both increased significantly, peaking in the infected fish at 9 and 16 d.p.i., respectively. The specific VN antibody response was similar to that described by McLoughlin *et al.* (1996), with 60% of fish producing specific neutralizing antibodies by 14–16 d.p.i. and all the fish seroconverting by 21 d.p.i., with tissue virus clearance in the majority of fish by 28 d.p.i. This suggests that specific immune responses are very important in the clearance of virus from the fish. There have been no reported cases of recurrence of PD or SD in previously infected populations, indicating that protection following natural infection is adequate for a complete production cycle.

A recent study by Jorgensen *et al.* (2007) using SAV 1 examined aspects of the interaction between SAV and the cellular immune response. It was found that the virus did not directly activate the interferon-induced Mx promoter of the gene encoding the antiviral Mx gene, and indeed interfered with interferon-α/β signalling. These findings suggest that SAV can antagonize the action of interferon to circumvent antiviral defence mechanisms.

The first reported vaccine trial was carried out by McLoughlin (1999). A simple formalin-inactivated (SAV 1) viral vaccine for PD was prepared and injected i.p. into Atlantic salmon parr, which were then experimentally challenged i.p. with live SAV 1, 4 weeks after vaccination. The results showed 100% protection in vaccinated fish, with no lesions observed and no virus re-isolated from pre- or post-challenge

fish in this group, whereas tissue virus and typical PD lesions were noted in all the unvaccinated controls.

Lopez-Doriga *et al.* (2001) also showed that Atlantic salmon parr immunized with binary ethyleneimine (BEI)-inactivated cultured SAV 1 virus were protected against cohabitant challenge with the wild-type (tissue homogenate) virus at 8 weeks post-vaccination. This also indicated that a culture-adapted isolate of SAV 1 could be used successfully to protect against wild-type virus and therefore could have potential use as an inactivated vaccine against PD.

A commercial PD vaccine has been in development since 1994. Initial extensive trials started in 1997 with a prototype SAV 1 antigen incorporated in a multivalent salmonid vaccine. It gave mixed results, as it appeared that the SAV 1 antigen was less efficacious in the presence of the other antigens in that vaccine (Christie *et al.*, 1999). A two-stage vaccination strategy has now been tested in both laboratory and commercial field trials in an attempt to protect salmon from PD. Moriette *et al.* (2006) reported on the development of a recombinant SAV 2 live vaccine which gave full protection from wild-type SAV 2 infection for 5 months in rainbow trout. These studies suggest that the development of an effective vaccine to protect salmonids from SAV diseases should be possible.

Prophylaxis and Control

Clinical disease will occur only where a number of factors including viral challenge load, stress, temperature and possibly other contributing factors interact. Therefore, it may be difficult for most sites in endemic areas to avoid SAV infection, so they must minimize its impact by reducing stress through careful management and good hygiene methods. Some risk factors that have been identified include sea-site to sea-site movements of fish, which are common in Ireland (McLoughlin *et al.*, 2003; Rodger and Mitchell, 2007), and sea-site to harvest movements using well boats, which are common in Norway. The SAV status of the fish can be checked by testing for viraemia, which will detect recent infection, and/or RT-PCR techniques, which will detect recent and more chronic infections. SAV antibody detection will also confirm a previous SAV challenge. The transport of viraemic fish obviously stresses the fish but can also be a source of infection for adjacent farms. Well-boat and transporter cleaning and disinfection are also critical to control the spread of all infectious agents. As with other infectious diseases, the efficient removal and safe disposal of dead fish may reduce the viral challenge. Biosecure slaughter methods and safe disposal of offal and effluent are also keys to minimizing the risk from these processes.

Recent work by Graham *et al.* (2007d,e) on the physiochemical properties of SAV 1 has indicated that the virus is sensitive to high and low pH, high temperatures (composting/ensiling) and common virucidal disinfectants. It can survive for more than 2 months in sterile seawater at low temperatures (4°C), which means the virus could remain on farms or be transferred between adjacent farms without direct or indirect human or animal intervention.

Good sea lice control is desirable, not only for the health and welfare of the fish, but because sea lice may act as reservoirs and/or vectors, as there is some evidence that they can carry SAV (Weston *et al.*, 2002; Karlsen *et al.*, 2005). It is important to note that alphaviruses are arboviruses and most of them are maintained in nature by a biological transmission cycle between susceptible vertebrate hosts and haematophagous arthropods, which are usually ticks or mosquitoes in terrestrial transmissions. An alphavirus known as the southern elephant seal (SES) virus was isolated from the elephant seal louse (*Lepidophthirus macrorhini*) (a species-specific louse) in Australasia and, given the high SES virus seroprevalence in the seal population, the authors suggested that the SESV was transmitted by the lice (Linn *et al.*, 2001). While it has been shown that SAV infections can be transmitted without the aid of an insect vector (McLoughlin *et al.*, 1996), further work is required to determine the potential role of sea and freshwater lice in SAV infections.

Dietary management has also been applied to reduce stress and aid recovery. Many farms withhold feed from all fish on site for 5–10 days on suspicion of PD, as there is some circumstantial evidence that this reduces the impact and losses. However, in our experience, it may take 2–3 months for all pens on a site to become infected and this strategy may be causing unnecessary losses in production due to days off feed (Crockford *et al.*, 1999). Indeed, in one pen, it can take months from the first identification of clinical symptoms to the actual 'outbreak', if indeed infection does not remain subclinical (Graham *et al.*, 2007a). If possible, dietary management should be on a cage-by-cage basis to optimize any effect and reduce consequent growth penalties. It is also important that affected fish do not get too hungry, as frantic feeding behaviour may exacerbate cardiac and skeletal lesions and increase mortality (Rodger *et al.*, 1991). Additional vitamin E and C in the diet, which are excellent antioxidants, may aid tissue repair and recovery (Raynard *et al.*, 1991; McCoy *et al.*, 1994).

Pancreatic enzyme replacement therapy, as used in pancreatitis in dogs, has been evaluated in two different field trials, with conflicting results; one indicating a growth benefit and the other showing no significant impact (Rodger *et al.*, 1995; Grant, 2008). Given that the pancreas can recover quite quickly, the economics and practicality of pancreatic enzyme replacement may not be cost-effective. The strategic use of immunostimulants and immunomodulators is currently being investigated.

Codes of practice have been drawn up in Scotland and Norway to encourage best procedure in salmon production and they contain very useful general guidance on the avoidance and mitigation of infectious diseases in salmonid species (Aunsmo *et al.*, 2005; Anon., 2006).

Conclusions and Recommendations for Future Studies

Much progress has been made in advancing the knowledge of SAV in the past 5 years, particularly in relation to pancreas disease in Atlantic salmon during the marine phase of production, but there still remain a number of areas which require further research and development.

The prevalence of infection and disease due to SAV in both Europe and the rest of the world remain poorly defined. Diagnostic tools, including serological and molecular methods, are now available to facilitate these investigations. While Ireland, Norway and Scotland have taken the lead in this area, there is a need for additional data, particularly from the larger industries in Scotland and Norway. Such information could also be a starting point for a full economic appraisal of the consequences of SAV salmon production. Elsewhere in Europe, the situation in freshwater trout production remains largely unknown, despite recent progress (Bergmann *et al.*, 2005; Graham *et al.*, 2007c). Outside Europe, there is little information available, including North America, where there had been an early description of PD (Kent and Elston, 1987) and a more recent report of isolation of a Togavirus from farmed Atlantic salmon (Kibenge *et al.*, 2000). Despite the size of the salmon farming industry in South America, there is also little information on the SAV status. There is also a requirement for detailed epidemiological studies to identify risk factors for both the introduction of infection on to sites and for the development of clinical disease on these sites, so that rational, scientifically valid control and mitigation strategies can be developed.

Improved diagnostic methods including the development of immunohistochemical and molecular *in situ* techniques for the detection of antigen/genetic material in fixed tissues are essential to understand fully the pathogenesis of PD and SD and to differentiate between SAV diseases and similar pathologies such as HSMI and CMS. While RT-PCR is a useful screening tool, it cannot be used as a sole diagnostic indicator. It is important to make a sound diagnosis based on a combination of clinical signs, gross and histopathology, serology, viral isolation, plus molecular and/or immunohistochemical techniques.

While variations in virulence and pathogenicity of SAVs have already been demonstrated between salmonid species and between Atlantic salmon strains, further studies are required to determine the spectrum of virulence of different SAV subtypes and explore the molecular basis thereof. Additional work is also required on the genetics of resistance to SAV to determine how this can be best incorporated into breeding programmes. A search for potential vectors and reservoirs of SAV would enhance greatly our understanding of the natural history and the control of PD. Alongside this, there is a need for experimental studies to determine the susceptibility of both additional salmonid species (particularly those of Pacific origin) and non-salmonid species (potential reservoirs) to infection.

The role of vertical transmission and carrier fish also needs to be clarified. Much effort is being expended currently in searching for dietary and management strategies to ameliorate the impact of SAV infections in both the European Atlantic salmon and rainbow trout industries. Despite the challenges of conducting field studies in these areas, this work has the potential to reduce the impact of PD and SD outbreaks significantly, and should be continued.

Finally, it is only with the development of efficacious SAV fish viral vaccines and a customized vaccination strategy that significant control of these serious infectious diseases of salmonids will be achieved.

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7 Oncogenic Viruses and *Oncorhynchus masou* Virus

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Introduction

Skin tumours of fish are easily recognized, some of which have been known for centuries. Causes of fish tumour formation are varied and are often thought to be multifactorial. In more than half of all cases examined using electron microscopy and virological methods, virus or virus-like particles were found in tumour tissues. Oncogenicity has only been clearly demonstrated for herpesviruses isolated from tumours. Viruses found in skin tumours, tumour-associated and tumour-inducing, are listed in Table 7.1, and an oncogenic virus named *Oncorhynchus masou* virus is reviewed.

Due to their distinctive appearance and obvious pathological nature, tumours of fish have been recognized for centuries. Publications on fish tumours are widely scattered in scientific literature (Walker, 1969; Anders and Yoshimizu, 1994). The largest registry of tumours in lower animals was established at the Smithsonian Institution in Washington, DC, USA, in 1965. To date, benign epidermal papilloma to metastatic melanomas and hepatocellular carcinomas in more than 300 species of fish have been registered. Depending on the season and geographical location, certain types of skin tumours may occur in high prevalence in wild fish such as European eel (*Anguilla anguilla*), dab (*Limanda limanda*), European smelt (*Osmerus eperlanus*) and northern pike (*Esox lucius*) from the north-eastern Atlantic coastal areas, while the occurrence of 'carp-pox' lesion in cultured cyprinids has decreased in importance. Tumours with a suspected viral aetiology are well documented in mammals, birds, reptiles and fish. For fish, a viral aetiology of papilloma was first suggested by Keysselitz (1908). Due to the frequent epidemic occurrence of fish tumours, many scientists have suggested an infectious viral aetiology (Winqvist *et al.*, 1968; Walker, 1969; Mulcahy and O'Leary, 1970; Anders, 1989; McAllister and Herman, 1989; Lee and Whitfield, 1992; Anders and Yoshimizu, 1994), even though they could not always demonstrate viral particles. In these cases, evidence was usually based on the exclusion of other potential causative factors. From a detailed review of the scientific literature, it is concluded that viruses play an important role in the induction of skin tumours of fish. In benign tumours, such as epidermal hyperplasia, papilloma and fibroma, these were mostly herpesviruses and, less frequently, retro-, papova- or adenovirus (Table 7.1). In malignant forms such as sarcomas and lymphosarcomas, there was evidence of the involvement of retroviruses (Table 7.2). Typically, the tumours had just one viral

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type; in rare cases, different skin tumour types associated with different viruses occurred in the same specimen (Anders and Yoshimizu, 1994).

The significance of these viruses and virus-like particles for tumour induction is mostly speculative. So far, oncogenicity has been clearly demonstrated for only herpesviruses isolated from masu salmon (*O. masou*) and Japanese Asagi carp (*Cyprinus carpio*) (Table 7.3). Although in certain other cases tumour formation could be induced experimentally by inoculation of cell-free filtrates and/or live tumour cells (Table 7.4), attempts to isolate viruses in cell culture have been unsuccessful. For two herpesviruses, pathogenicity and oncogenicity have been clearly verified by successful isolation of the causative virus in cell culture and fulfilment of River's postulates. So far, nothing is known about possible oncogenes of these viruses. One of them is *Oncorhynchus masou* virus (OMV). This agent is a salmonid herpesvirus first isolated in 1978 from ovarian fluids of masu salmon in Hokkaido, Japan (Kimura *et al.*, 1981a). The virus has since been isolated frequently from mature masu salmon. OMV

Table 7.1. Naturally occurring fish skin tumours associated with viruses or virus-like particles.

Host species	Tumour type	Associated virus or VLP	Proof by	Source
Anguilla anguilla	H	Herpesvirus	T	Bekesi *et al.* (1986)
Oncorhynchus masou	P	Herpesvirus (OMV)[a]	I, E, R	Kimura *et al.* (1981a,b)
Salmo salar	H, P	Retro-VLP	T	Carlisle and Roberts (1977)
	P	Herpes-VLP	T	Shchelkunov *et al.* (1992)
Salvelinus namaycush	H(?)	Herpesvirus	T, E	McAllister and Herman (1989)
Osmerus eperlanus	H	Herpesvirus	T	Anders and Möller (1985)
	H	Retro-VLP	T	Anders (1989)
Esox lucius	H	Herpesvirus	T	Yamamoto *et al.* (1984)
Europe	H	Retro-VLP	T	Winqvist *et al.* (1968)
North America	H	Retro-VLP	T	Papas *et al.* (1976)
Cyprinus carpio	P	Herpesvirus (CHV)[a]	T	Schubert (1964)
			I, E, R	Sano *et al.* (1985a,b)
Leuciscus idus	H, P	Herpesvirus	T	McAllister *et al.* (1985)
Catostomus commersoni	P	Retro-VLP[b]	T	Sonstegard (1973, 1977)
Xiphophorus hybrids	M	Papova-VLP	T, Pr	Kollinger *et al.* (1979)
Ictalurus nebulosus	P	VLP	T	Edwards and Samsonoff (1977)
Silurus glanis	H, P	Herpesvirus	T	Bekesi *et al.* (1981)
Gadus morhua	H	Adeno-VLP	T	Jensen and Bloch (1980)
Stizostedion vitreum	H	Herpesvirus	T, I	Kelly *et al.* (1980, 1983)
	H	Retro-VLP	T	Walker (1969)
Sparus aurata	P	VLP	T	Gutierrez *et al.* (1977)
Agonus cataphractus	F	Retro-VLP (lentivirus group)	T	Anders *et al.* (1991)
Limanda limanda	H, P	Adeno-VLP	T	Bloch *et al.* (1986)
Pseudopleuronectes americanus	H, P	Papova-VLP	T	Emerson *et al.* (1985)

P, epidermal papilloma; H, epidermal hyperplasia; M, melanoma; F, fibroma; T, transmission electron microscopy; I, isolation from tumour tissue; E, successful experimental infection; R, re-isolation; Pr, activation of virus by tumour promoter; [a]proven oncogenicity; [b]evidence for reverse transcriptase activity.

Table 7.2. Naturally occurring malignant fish skin tumours associated with viruses or virus-like particles.

Host species	Tumour type	Associated virus or VLP	Proof by	Source
Esox lucius	S	Retro-VLP	T	Winqvist *et al.* (1968)
	L	Retrovirus	Rt	Papas *et al.* (1976)
Gymnothorax funebris	L	Retrovirus	Rt	Buck *et al.* (2001)
Stizostedion vitreum	S	Retro-VLP	T, Rt	Yamamoto *et al.* (1976)

S, sarcoma; L, lymphosarcoma; T, transmission electron microscopy of tumour tissue; Rt, evidence of reverse transcriptase activity.

Table 7.3. Experimentally induced tumours in fish.

Host species	Tumour type	Oncogenic virus	Source
Oncorhynchus masou	P	OMV	Kimura *et al.* (1981a,b)
Oncorhynchus kisutch	P	OMV	Horiuchi *et al.* (1989)
Oncorhynchus keta	P	OMV	Kimura *et al.* (1981a,b, 1983a)
Oncorhynchus mykiss	P	OMV	Suzuki (1993); Furihata *et al.* (2003)
Cyprinus carpio	P	Herpesvirus	Sano *et al.* (1985a,b)

P, epidermal papilloma.

Table 7.4. Experimentally induced malignant skin tumours in fish.

Host species	Tumour type	Source
Esox masquinongy	L[a]	Sonstegard (1976)
Esox lucius	L[a,c]	Sonstegard (1976); Papas *et al.* (1977)
Salvelinus namaycush	H	McAllister and Herman (1989)
Scardinius erythrophthalmus	S[c]	Hanjavanit and Mulcahy (1989)
Stizostedion vitreum	L	Martineau *et al.* (1990)
Pomacentrus partitus	N[b]	Schmale and Hensley (1988)

L, lymphosarcoma; H, epidermal hyperplasia; S, squamous cell carcinoma; N, neurofibroma. [a]Inoculation of cell-free filtrates; [b]inoculation of homogenized tumour tissue; [c]inoculation of live tumour cells.

is pathogenic to masu salmon, chum salmon (*O. keta*), kokanee salmon (*O. nerka*), coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*). Infected fish become anorexic and show exophthalmia and/or petechiation on the body surface. Cumulative mortality ranged from 80 to 100% in masu, chum and kokanee salmon and from 29 to 39% in coho salmon and rainbow trout (Kimura *et al.*, 1983a). Beginning at about 4 months post-infection and persisting for at least 1 year, 12–100% of surviving chum, coho and masu salmon and rainbow trout developed epithelial tumours around the mouth (Kimura *et al.*, 1981b). The tumours were characterized as epidermal papillomas and basal cell carcinoma. Several layers of epithelial cells in a papillomatous array were supported by a fine connective tissue stroma. Abundant mitotic figures suggested a highly proliferative nature. Tumours appearing on the caudal fin, gill cover, body

surface, corneas of the eye and kidney showed characteristics similar to those of the mouth (Yoshimizu *et al.*, 1987). Electron microscopy revealed that the tumour cells had a typical neoplastic feature of variability in nuclear size and loose intercellular connections. However, OMV particles have never been found in either the nuclei or cytoplasm of tumour cells (Yoshimizu *et al.*, 1987).

The second is *Cyprinid herpesvirus* (CyHV-1). Schubert (1964) first visualized this virus using electron microscopy. It is associated with the earliest known skin tumour of fish, the so-called 'carp-pox' papilloma of common carp. After several unsuccessful attempts to isolate the virus in cell culture (Schubert, 1964), it was recovered finally from naturally occurring papillomas of Japanese fancy carp. The isolate was virulent for carp fry following exposure by immersion. Cumulative mortality was 85.7% in 2-week-old common carp and 20% in 4-week-old fancy carp. The virus was re-isolated from all moribund fish and all survivors. At 5–6 months post-infection, 83% of surviving common and fancy carp fry developed epidermal papillomas on fins, body surface, mandibles and the sites of inoculation. Experimental infection of adult fish led to 13% tumour incidence in mirror carp and 10% in fancy carp. In 50% of the cases, CyHV-1 was re-isolated successfully from tumour-infected fry (Sano *et al.*, 1985a,b).

There is strong evidence for potential oncogenicity of one additional herpesvirus and two retroviruses, which have not yet been isolated in cell culture but which have been associated with successful experimental transmissions of infections.

First is epithelial hyperplasia of lake trout (*Salvelinus namaycush*). Bradley *et al.* (1989) and McAllister and Herman (1989) reported on epizootic mortalities among hatchery-reared fingerling and yearling lake trout. Epithelial hyperplasias on jaws, the body surface or inside the mouth were found consistently to be associated with the disease. A putative herpesvirus was observed in tumour tissue by electron microscopy. Experimental transmission of the infection was successful by cohabitation with diseased fish, by exposure to water from tanks holding diseased fish and by bath challenge using cell-free filtrates of epidermal hyperplastic tissue.

Second is northern pike (*E. lucius* L.) and muskellunge (*E. masquinongy* Mitchill) lymphosarcoma. Malignant lymphoma or lymphosarcoma is the most common neoplasm identified in esocid species, particularly in the northern pike (Mulcahy, 1963) and the muskellunge (Sonstegard, 1976). The prevalence of these neoplasms in northern pike has approached 12.5% in Ireland (Mulcahy, 1963), 20% in North America (Sonstegard, 1975) and 10% in Sweden (Thompson, 1982). The neoplasm usually presents as cutaneous lesions involving jaws, flank and fin base, but may also involve internal organs such as kidney, spleen and thymus (Mulcahy, 1963; Mulcahy and O'Leary, 1970). Based on light and electron microscopy examination, together with limited immunohistochemical procedures, it is suggested that the neoplastic cells resemble stem cells (Mulcahy *et al.*, 1970). There is evidence for a viral and in particular retroviral aetiology, as the disease has been transmitted in Irish pike by cell-free filtrates (Mulcahy and O'Leary, 1970; Sonstegard, 1976). Virus particles and a temperature-sensitive reverse transcriptase activity have been reported in fractionated tumour cells from Canadian pike (Papas *et al.*, 1976, 1977). Later, it was confirmed that lymphoma in northern pike had a retroviral aetiology with the description of a retrovirus-like particle from tumour tissue using electron microscopy (Winqvist *et al.*, 1973). In addition, neoplasm was induced in healthy pike kept in the same water as tumour-bearing pike (Ljungberg, 1976), through the detection of reverse transcriptase activity and from the detection of cytoplasmic particulate fractions (CPF) from the tumour (Papas *et al.*, 1976). Microscopically, the neoplasm is non-encapsulated, well-defined nodular masses distributed within the tissues of each organ. The principal histopathological features of the tumour are: type of cells, size, shape, staining and pattern of growth

(typical of lymphoma). Using electron microscopy, Banfield *et al.* (1976) showed cells of the malignant lymphoma of the northern pike from Canada contained cylindrical structures with central cores of cytoplasmic elements and walls composed of lamellae in a spiral arrangement, separated by ribosome-like particles or by membranes. These cylindroids were similar to structures in the cells of certain human lymphomas and leukaemia.

Third is the so-called 'dermal sarcoma' of walleye (*Stizostedion vitreum*). Martineau *et al.* (1990, 1991a,b) and Bowser *et al.* (1990) also successfully transmitted the dermal sarcoma of walleye which was considered benign. It is notable that successful experimental induction of tumours is associated mostly with herpesviruses, whereas formation of malignant forms seems to be restricted to the involvement of retroviruses. It has not yet been possible to isolate a tumour-associated retrovirus in cell culture. However, in several cases, experimental induction of malignant skin tumours using cell-free filtrates or live tumour cells was successful, thus suggesting a viral aetiology (Table 7.4). Furthermore, several research groups are circumventing problems with cell culture by taking a molecular approach (Zhang *et al.*, 1996; Getchell *et al.*, 2002; Rovnak *et al.*, 2005; Eizert *et al.*, 2008; Quackenbush *et al.*, 2009).

Viruses visualized using electron microscopy include herpesvirus, adenovirus, papovavirus and retrovirus members, as well as unidentified particles of a doubtful viral nature. A total of 22 apparently distinct viruses, including OMV and CyHV-1, have been described from cartilaginous and bony fish (Hedrick and Sano, 1989).

Oncorhynchus masou Virus (OMV)

Introduction

Distribution of salmonid herpesvirus was known in the USA and Japan. Herpesviruses isolated in the USA were classified as serotype 1 (SaHV-1) and in Japan as serotype 2 (SaHV-2). The reference strain of SaHV-1 is *Herpesvirus salmonis* and SaHV-2 is *Oncorhynchus masou* virus (OMV) strain OO-7812 (Yoshimizu *et al.*, 1995). A herpesvirus infection of salmonid fish in Japan was first described by Sano (1976) on isolation from moribund kokanee salmon in Towada Lake, in the northern part of Honshu in mainland Japan. Subsequently, in 1978, a herpesvirus was isolated from the ovarian fluid of mature masu salmon cultured in Hokkaido. The virus characterized as an *onco*genic virus was isolated from land-locked masu salmon (Kimura *et al.*, 1980a,b, 1981a). Members of the genus *Oncorhynchus* belong to the Pacific salmon and are mainly anadromous, but some small populations have been isolated and land-locked. Following the discovery of OMV, many strains of herpesvirus that can be neutralized with antiserum against OMV have been isolated from cultured and wild salmonid fish in the northern part of Japan (Yoshimizu *et al.*, 1993). Since 1988, OMVD has become a major problem in pen culture of coho salmon in the Tokoku district (Horiuchi *et al.*, 1989) and since 1991 OMV has been found in pond cultures of rainbow trout in Hokkaido and the central part of Japan (Suzuki, 1993; Furihata *et al.*, 2003). Synonyms include the nerka virus in Towada Lake, Akita and Aomori Prefecture (NeVTA), yamame tumour virus (YTV), *Oncorhynchus kisutch* virus (OKV), coho salmon tumour virus (CSTV or COTV), coho salmon herpesvirus (CHV), rainbow trout kidney virus (RKV) and rainbow trout herpesvirus (RHV). This virus is pathogenic and more significantly oncogenic for the young masu salmon and several other salmonid fish. OMV is widely distributed in the northern part of Japan. OMV disease (OMVD) causes hepatitis and oncogenic and/or skin ulcer conditions. The main susceptible fish species are kokanee salmon, masu salmon, coho salmon and rainbow trout. Economic losses caused by this virus are significant, especially in kokanee salmon, coho salmon and rainbow trout (Yoshimizu and Nomura, 2001).

Geographical Distribution and Host Range

Herpesvirus isolated from kokanee salmon

High mortality has been observed among the fry of kokanee salmon (land-locked *O. nerka)* from June to September of every year since 1970. Mortality may reach over 80% for the 3-month period. Clinical signs in infected fish include a darkening in body colour, sluggish behaviour and loss of appetite. From these moribund fish, a syncytium-forming virus was isolated in RTG-2 cells incubated at 10°C in 1972 and 1974. The virus was classified as a member of Herpesviridae and it was named the nerka virus in Towada Lake, Akita and Aomori Prefecture (NeVTA) (Sano, 1976).

Herpesvirus isolated from masou salmon

In 1978, a herpesvirus was isolated from the ovarian fluid of an apparently normal mature masu salmon, cultured in the Otobe salmon hatchery in Hokkaido. This virus was named *Oncorhynchus masou* virus, after the scientific name of the host fish and its oncogenicity (Kimura *et al.*, 1981a,b). The general properties of OMV are similar to those of *H. salmonis* and NeVTA, but it differs in virion size and its optimal growth temperature. It is also distinct from *H. salmonis* with respect to its virus-induced polypeptide patterns, serological properties and polymerase chain reaction (PCR) (Kimura and Yoshimizu, 1989; Aso *et al.*, 2001). OMV is pathogenic and, more significantly, oncogenic for masu salmon and several other salmonid fish (Kimura *et al.*, 1981a,b). One-month-old kokanee salmon are most sensitive to the virus. Masu and chum salmon also have high susceptibility, while coho salmon and rainbow trout are less susceptible to OMV infection (Tanaka *et al.*, 1984). The incidence of tumour-bearing fish reached more than 60%. Epithelial tumours were found on 12–100% of the surviving chum, coho and masu salmon and rainbow trout, beginning at about 4 months and persisting for at least 1 year post-infection (Yoshimizu *et al.*, 1987). Since its discovery in 1978, at the Otobe Salmon Hatchery, OMV has been isolated from the ovarian fluid and neoplastic tissue of mature masu salmon collected from other places. In 1981, a similar herpesvirus was isolated from the tissues of a basal cell carcinoma that developed on the mouthpart of yamame (another name is masu salmon) cultured at Koide Branch, Niigata Prefectural Inland Fisheries Experimental Station. This virus was named yamame tumour virus (YTV) (Sano *et al.*, 1983). Serologically, Yoshimizu *et al.* (1995) confirmed NeVTA, OMV and YTV as being the same virus. From the results of the comparison of the DNA homologies, OMV and YTV were classified as the same virus and NeVTA was classified as being similar yet distinct from these two viruses (Eaton *et al.*, 1991).

Herpesvirus isolated from coho salmon

Since 1988, herpesvirus has been isolated from the liver, kidney and developing neoplasm in pond- and pen-cultured coho salmon (Kimura and Yoshimizu, 1989). Affected fish show the following disease signs: ulcers on their skin, white spots on their liver and neoplastic tissues around their mouthpart or body surface. Coho salmon culture is economically damaged by this disease. The herpesviruses isolated from coho salmon were tentatively named as coho salmon tumour virus (CSTV) by Igari *et al.* (1991), *O. kisutch* virus (OKV) by Horiuchi *et al.* (1989), coho salmon tumour virus (COTV) by Kimura and Yoshimizu (1991) and coho salmon herpesvirus (CHV) by Kumagai *et al.* (1994). All of these viruses were neutralized by anti-OMV or NeVTA rabbit serum (Yoshimizu *et al.*, 1995), and the oncogenicity of CSTV, OKV and COTV was confirmed by experimental infection. In addition, restriction endonuclease profiles of CSTV were the same as those of NeVTA and YTV (Igari *et al.*, 1991). CHV showed strong pathogenicity to coho salmon.

Herpesvirus isolated from rainbow trout

Massive mortality has occurred among 1-year-old rainbow trout in pond cultures since 1992 in Hokkaido. The diseased fish exhibited almost no clinical signs. Some fish did manifest ulcerative lesions on their skin. Internally, intestinal haemorrhage and white spots on the liver were observed. No bacterial, fungal or parasitic agents were found and the herpesvirus was isolated from the kidney, liver and ulcerative skin tissues. The rainbow trout culture industry experienced serious economic losses due to this disease, since rainbow trout of marketable size were affected and died. This herpesvirus was tentatively named rainbow trout kidney herpesvirus (RKV) by Suzuki (1993). The virus was neutralized with anti-OMV rabbit serum and its main characteristics were the same as OMV. RKV showed strong pathogenicity to marketable-sized rainbow trout and masu salmon (Sung *et al.*, 1996a,b).

Epizootics occurred in cultured rainbow trout weighing 12 g to 1.5 kg at 18 fish farms from February 2000 to January 2001 in Nagano Prefecture, Japan. A virus was isolated from diseased fish in RTG-2 cells with a CPE characterized by syncytia. High infectivity titres about 10^8 $TCID_{50}$/g were demonstrated in the main internal organs and multiple necrotic foci were observed in the liver. The virus was identified as OMV using serological tests and PCR. Based on these results, the epizootic was diagnosed as OMVD. In more than 80% of cases, the outbreaks were linked to introductions of live fish (Furihata *et al.*, 2003).

Roots of OMV

Six species of mature salmonid fishes (29,032 females) were collected (1978–2002) to survey the incidence of this virus in Hokkaido and the northern part of Honshu (Yoshimizu *et al.*, 1993; Kasai *et al.*, 2004). Herpesvirus was isolated from masu salmon at all the investigated sites, with the exception of one hatchery. All of the isolates were neutralized with anti-OMV rabbit serum (Yoshimizu *et al.*, 1993). Based on our epizootiological study, the route of OMV in Japan was assumed to be along the Japan Sea coast of Hokkaido and the presumed original host species was masu salmon. In the 1960s, eggs of masu salmon were collected from the rivers of the Japan Sea coast of Hokkaido and transported to Honshu Island, the mainland of Japan. With unrestricted fish movement, the virus spread to several places in Honshu, where the first cancer disease of masu salmon was observed (Kimura, 1976), and also in Hokkaido, where OMV had already been detected. Subsequently, coho salmon and rainbow trout were cultured in the same water systems where masu salmon was cultured. Coho salmon might be infected with OMV at fry stage in fresh water because tumour tissue was found around the mouth of pen-cultured coho salmon; the hatchery from where coho salmon was transplanted to pen had a history of OMVD (Yoshimizu and Nomura, 2001).

The Disease Agent

Biophysical and biochemical properties

The biophysical and biochemical properties of OMV are shown in Table 7.5. At a near optimal incubation of 15°C, OMV shows distinctive CPE in 5–7 days, rounded cells followed by syncytium formation and eventual lysis of RTG-2 and other salmonid cell lines (Fig. 7.1a and b). The cells from non-salmonid species are refractory to infection (Yoshimizu *et al.*, 1988b). The maximum titre of culture-grown virus is about 10^6 $TCID_{50}$/ml, with some variation depending on the cell lines. OMV is heat-, ether- and acid (pH 3)-labile and does not haemagglutinate human O-cells. It is completely inactivated by ultraviolet (UV) irradiation with $3.0\times10^3 \mu W{\cdot}s/cm^2$. In the presence of 50$\mu$g/ml of the pyrimidine analogue, 5-iododeoxyuridine (IUdR), replication is inhibited. Replication of OMV is also inhibited by anti-herpesvirus agents such as phosphonoacetate (PA), acyclovir (ACV),

Table 7.5. Characteristics of *Oncorhynchus masou* virus (strain OO-7812).

Size (nm)	Nucleocapsid	115 ± 7~14
	Enveloped virion	200 ± 24 × 240 ± 23 nm
Capsomere		162
Infectivity titre ($TCID_{50}$/ml)	RTG-2 cells	$10^{5.3\sim5.8}$
	Masu and chum salmon	$10^{4.8\sim5.8}$
	Rainbow trout	$10^{8.3\sim8.8}$
Susceptible cells	All salmonid cell lines	
Refractory cells	Non-salmonid cell lines	
CPE morphology	Rounded	Syncytium formation
Optimum temperature (°C)	Growth	15
	DNA polymerase	25
Inhibition or inactivation	Temperature	50°C 5 min, 60°C 1 min
	Ether and pH 3	Yes
	Acyclovir 2.5 μl	Yes
Target organ	1 month old	Kidney
	3 months old	Liver
Preservation	0°C	1 week
	–20°C	2 weeks
	–80°C	More than 30 years
	–196°C (liquid nitrogen)	More than 20 years

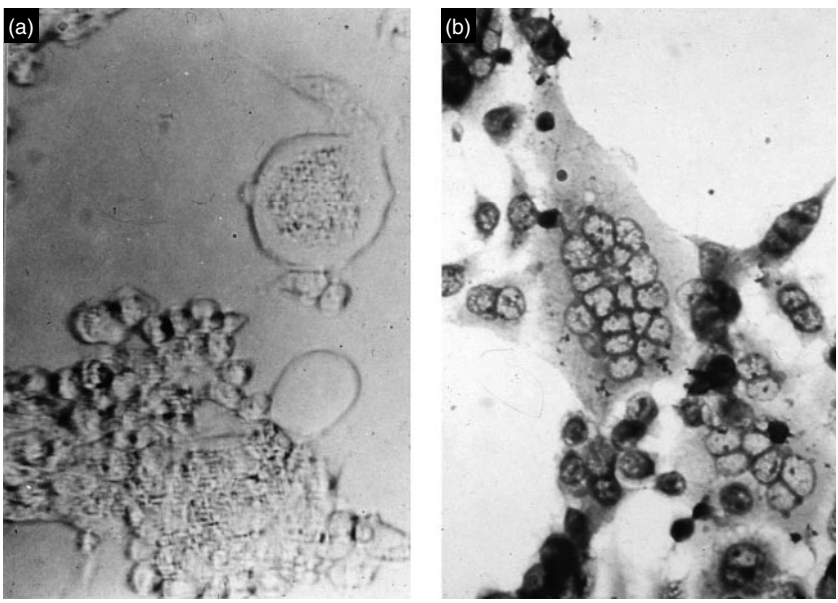


Fig. 7.1. Cytopathic effects produced by OMV in (a) RTG-2 and (b) CHSE-214 cells incubated at 15°C for 9 days: (a) not fixed; (b) May Grünwald Giemsa stain.

(*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU), and 1-β-D-arabinofuranosylcytosine (Ara-C), which is caused by the inhibition of DNA polymerase induced by OMV (Kimura *et al.*, 1981a, 1983b,c, 1987; Suzuki *et al.*, 1992).

Electron microscopy of infected cells reveals that the intranuclear hexagonal capsids have a diameter of 115 nm. Abundance of budding, enveloped virions, 200 × 240 nm in diameter, was also observed

on the surface and inside cytoplasmic vesicles (Fig. 7.2). The calculated number of capsomere of negatively stained virions is 162 (Fig. 7.2b). These features confirm that OMV is a herpesvirus. The optimal growth temperature of OMV is 15°C. Viral growth was not observed at 20°C or higher, although replication was observed at 18°C. This psychrophilic nature of OMV differs highly from channel catfish herpesvirus and herpesvirus found in amphibians (Kimura *et al.*, 1981a). OMV is designated as salmonid herpesvirus 2 (Yoshimizu *et al.*, 1995).

Viral protein and gene

The general properties of OMV are similar to those of *H. salmonis*, salmonid herpesvirus 1; however, OMV differs in virion size and in the optimal growth temperature. Furthermore, OMV is different from other known fish herpesviruses with respect to the virus-induced polypeptide patterns; 34 polypeptides appearing in OMV-infected cells are designated as virus-specific. These polypeptides possess molecular weights ranging from 19,000 to 227,000. However, *H. salmonis* induced 25 polypeptides with molecular weights ranging from 19,500 to 250,000. CCV induced 32 polypeptides (Dixon and Farber, 1980), and the polypeptide patterns of CCV are also distinct from those of OMV. Differences in the electrophoretic migration of two of 34 OMV-specific polypeptides led to the classification of 12 OMV strains into six groups (Kimura and Yoshimizu, 1989).

Restriction endonuclease cleavage patterns of OMV DNAs were different from those of *H. salmonis*. Seven representative OMV strains, which were isolated from ovarian fluid and tumour tissue of cultured as well as wild masu salmon in Hokkaido and Aomori Prefecture, were analysed with the restriction endonuclease. The restriction patterns of OMV strain DNAs were divided into four groups. Restriction profiles of high passage strains were different from those of low passage strains when digested with *Bam*HI, *Hin*dIII and *Sma*I. However, no difference was observed between the high and low passage viral DNA with *Eco*RI (Hayashi *et al.*, 1987). By using 32P-labelled DNA of standard OMV (strain OO-7812) as a probe, most of the fragments of other OMV strain DNAs were hybridized (Gou *et al.*, 1991a,b).

DNA polymerase activities were surveyed in the tumour tissue and normal tissue of masu salmon. High activity of DNA polymerase α was detected in the tumour tissue, but not in the normal tissue. This indicates that the tumour cells replicate prosperously. Viral DNA polymerase activity was detected in the tumour tissue only, indicating that OMV DNA should replicate there. DNA polymerase activity was of the same level in both tissues. This is the first evidence that herpesvirus DNA polymerase was detected in tumour tissue in association with herpesvirus (Suzuki *et al.*, 1992).

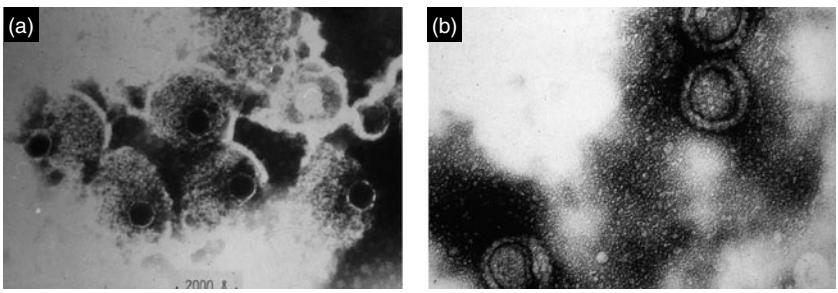


Fig. 7.2. Electron micrograph of negatively stained (a) enveloped virions (provided by Dr T. Sano) and (b) virions.

Serological relationship

The antigenic properties of OMV are distinctively neutralized by homologous antiserum, but not by the antiserum of the other salmonid viruses (e.g. IPNV, IHNV, CSV and *H. salmonis*). All 177 OMV strains isolated from ovarian fluid or tumour tissue of mature masu salmon in hatcheries located in various parts of northern Japan, collected from 1978 to 1986, were neutralized with anti-OMV OO-7812, a reference strain of OMV rabbit serum, and the ND_{50} ranged from 1:40 to 1:80 (Yoshimizu *et al.*, 1988b).

The 11 herpesvirus strains isolated from kokanee salmon (NeVTA), masu salmon (three strains of OMV and YTV), coho salmon (CSTV, COTV and two strains of OKV), rainbow trout (RKV and RHV) and *H. salmonis* were compared for their serological relatedness using serum cross-neutralization tests with polyclonal rabbit antiserum. The herpesvirus strains isolated in Japan were neutralized by antisera against these viruses and were found to be related closely to salmonid herpesvirus 2, reference strain OMV OO-7812 (Table 7.6). These strains, however, are clearly distinguished from salmonid herpesvirus 1 (Yoshimizu *et al.*, 1995).

Economic Importance of the Disease

Following the discovery of OMV, many strains of herpesvirus identified as OMV have been isolated from diseased kokanee salmon and tumour-inducing cultured and wild masu salmon in the northern part of Japan (Yoshimizu *et al.*, 1993). The mortality of kokanee salmon reached 80–100%. In the case of masu salmon, there are no reports of mortality caused by OMV, only induced tumour damaged for commercial trade. Since 1988, OMVD has become a major problem in pen cultures of coho salmon in the Tohoku district and coho salmon culture is economically damaged by this disease (Fig. 7.3a). From 1991, OMV has been found in pond cultures of rainbow trout in Hokkaido and, since 2002 (Fig. 7.3b), in the central part of Japan. Now, OMVD among kokanee salmon, masu salmon and coho salmon is regulated and controlled by the methods described below and OMVD outbreaks are observed in rainbow trout only. OMV kills commercial-sized rainbow trout and this disease is still a major problem for rainbow trout farms (Furihata *et al.*, 2003).

Diagnostic Methods

The agent's infectivity remains unchanged for at least 2 weeks at 0–5°C, but at –20°C 99.9% of infectivity is lost within 17 days. Virus isolation should be carried out using fish transported on ice to the laboratory (Yoshimizu *et al.*, 2005). For filtration of OMV, a 0.40 μm nucleopore filter (polycarbonate) is recommended because the cellulose acetate membrane filter traps virus particles. For the purpose of a virological survey of mature salmonid, ovary fluid is collected by the method described by Yoshimizu *et al.* (1985), with the addition of the same volume of antibiotic (Amos, 1985) and reacted at 5°C overnight. In the case of tumour tissues, the tissue is cut and disinfected with iodophore (50 mg/l, 15 min), then washed with Hank's BSS and transported with antibiotic solution to the laboratory. Tumour tissues must be prepared for the primary culture or co-culture with RTG-2 cells. After one subculture of primary culture cells, virus inspection of the culture medium should be carried out. Usually, RTG-2 cells are harvested and inoculated; a suitable incubation temperature is 15°C.

In the laboratory, rabbit serum or monoclonal antibody against OMV is used for a fluorescent antibody test (Hayashi *et al.*, 1993) and DNA probe is also used for detection of virus genome (Gou *et al.*, 1991b). PCR using an F10 primer, GTACCGAAACTCCGAGTC, and R05 primer, AACTTGAACTACTCCGGGG, amplified a 439 base-pair segment of DNA from OMV strains isolated from masu salmon, coho salmon and rainbow trout, and liver, kidney, brain and nervous tissues. Agarose gel profile of amplified DNA was able to

Table 7.6. Serological relationship of herpesvirus strains isolated from salmonid fish using the 1/r value calculated by a serum cross-neutralization test.

		Antiserum								
		OMV								
Species	Virus	I	II	III	YTV	NeVTA	COTV	OKV (M)	RKV	HS
Masu	OMV I	1.00	1.30	0.92	1.42	1.12	1.53	0.85	1.22	>3.16
	OMV II		1.00	0.80	1.00	1.22	1.47	0.85	0.67	>3.47
	OMV III			1.00	1.21	0.94	1.41	1.00	0.83	>3.47
	YTV				1.00	0.84	1.33	0.70	1.19	>5.69
Kokanee	NeVTA					1.00	1.39	0.83	0.89	>4.89
Coho	COTV						1.00	0.96	0.51	>3.16
	OKV (M)							1.00	0.83	>3.47
Rainbow trout	RKV								1.00	>3.47
Rainbow trout	*H. salmonis*									1.00

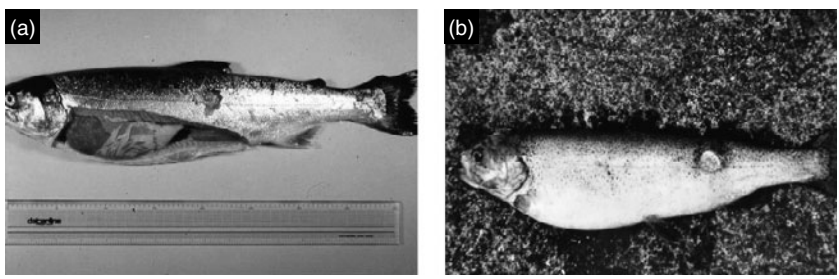


Fig. 7.3. (a) OMV infected coho salmon and (b) rainbow trout. White spot lesions on the liver and ulcerative skin lesions were observed. Photograph of coho salmon was provided by Dr A. Kumagai.

distinguish an OMV and *H. salmonis* (Fig. 7.4) (Aso *et al.*, 2001).

Presumptive diagnosis

To aid in the diagnosis of OMVD, certain key features such as life-cycle stage and species of fish, water temperature, disease signs and disease history of the facility and stock of fish are evaluated. To isolate OMV, tissue and reproductive fluids are examined by standard cell culture techniques. Processed specimens must be inoculated on to the rainbow trout gonad cells (RTG-2) or Chinook salmon embryo cells (CHSE-214). Cytopathic effect includes rounded cell and giant syncytium formation. Plaque assay procedures similar to those of Burke and Mulcathy (1983) and Kamei *et al.* (1987), which use a methyl cellulose overlay, are also used for isolation and enumeration of OMVD. Clinical signs, microscopic pathology, past history of OMVD and observation of typical CPE provide the best evidence for a presumptive diagnosis.

Confirmatory diagnosis

Confirmation of OMV is accomplished using serum neutralization tests with a polyclonal rabbit antisera or monoclonal antibodies. An antigen detection ELISA for OMV has

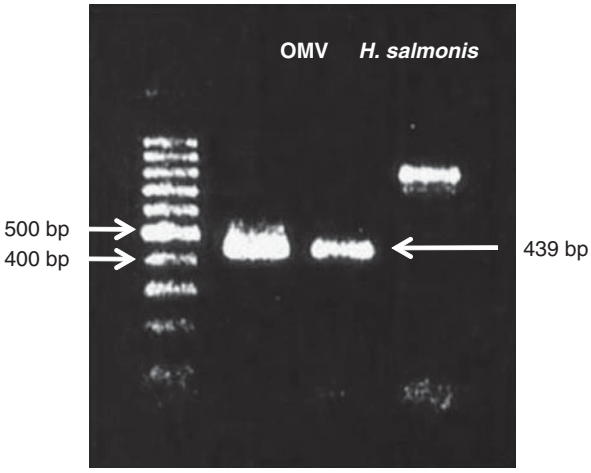


Fig. 7.4. Agarose gel profile of PCR products.

been reported. A FAT for OMV was developed using either polyclonal or monoclonal antisera. The FAT was specific and reached all isolates of OMVD tested and required less time to obtain a definitive diagnosis. Also, the PCR method has been accepted for confirmation of culture-grown OMV (Aso *et al.*, 2001).

Procedures for detecting subclinical infection

Detection of OMV in carrier fish is difficult but the virus replicates and appears in ovarian fluid at the mature stage. Antibody detection ELISA reporting by Kim *et al.* (2007) is available for epizootiological study.

Pathogenicity and Immunity

Pathogenicity and host range

Susceptibility of several salmonid fry to OMV has been studied experimentally by immersion in water containing 100 $TCID_{50}$/ml OMV at 10°C for 1 h. Compared with the five different salmonid fry, at the age of 1 month, kokanee salmon exhibited the greatest sensitivity and 100% of them died. Masu and chum salmon also exhibited high sensitivity at 87 and 83%, respectively. Coho salmon and rainbow trout were shown to be less sensitive to OMV infection at 39 and 29% mortality, respectively. Thus, the host range of OMV is wide in salmonid species (Fig. 7.5) (Kimura *et al.*, 1983a). Eight age groups of chum salmon (0, 1, 2, 3, 4, 5, 6, and 7 months old) were immersed under the same conditions. The cumulative mortality of just hatching chum salmon, observed in the ensuing 4 months, was 35%, but between 1- and 5-month-old fry it was more than 80%. In particular, at 3 months, the fry exhibited 98% mortality, which should be the greatest sensitivity. At 6 and 7 months, the fry's susceptibility was reduced and only 7 and 2% of fish had succumbed to the disease. There were no deaths among 8-month-old fingerlings that were immersed in a suspension of virus and additionally injected intraperitoneally with 200 $TCID_{50}$/fish. On the other hand, 1-month-old masu salmon fry were most sensitive and cumulative mortality reached 87%. In 3- to 5-month-old fry, cumulative mortality decreased from 65 to 24% (Kimura *et al.*, 1980a, 1983a).

Fish-to-fish transmission was accomplished by holding 5-month-old fry with fry that had been infected by immersion; the resulting mortality was similar to that observed as a result of original infection by immersion. From the evidence obtained, OMV is considered to be a virulent salmonid

virus and its influence on salmon culture should not be neglected. Clinical signs in infected fish are inappetence and exophthalmia, and petechiae on the body surface, especially beneath the lower jaw. Agonal or abnormal swimming behaviour has not been observed. Internally, the liver shows white spot lesions (Fig. 7.6a) and, in advanced cases, the whole liver becomes pearly white. In some cases, the spleen is swollen. The digestive tract is devoid of food (Kimura *et al.*, 1981a, 1983a).

Histopathology

The kidney of OMV infected 1- and 3-month-old masu salmon, 1-month-old coho salmon and 2-month-old chum salmon is the principal target organ for the virus, as judged by the severity of histopathological changes found in infected 1-month-old masu salmon. Necrosis of epithelial cells and kidney was observed in the early moribund specimens, while partial necrosis of the liver, spleen and pancreas was seen in later moribund

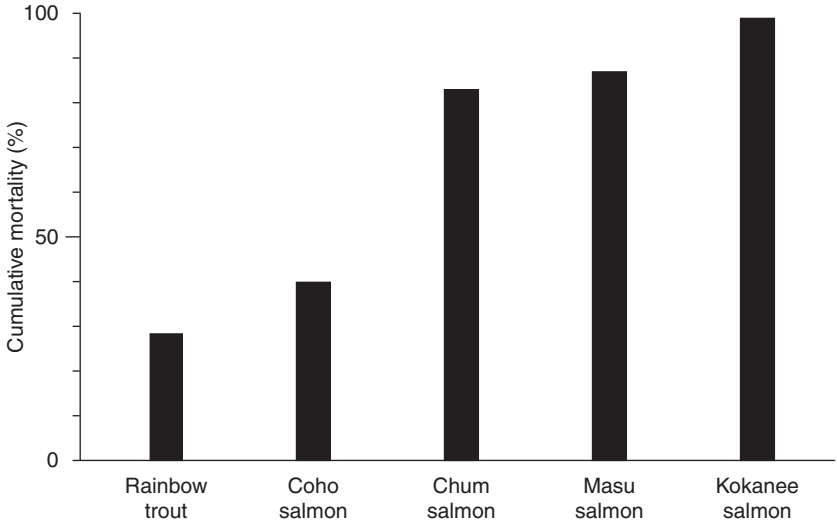


Fig. 7.5. Cumulative mortality of five species of salmonid fish – rainbow trout, coho, chum, masu and kokanee salmon – fry exposed to OMV. Exposing dose was 100 $TCID_{50}$/ml for 60 min.

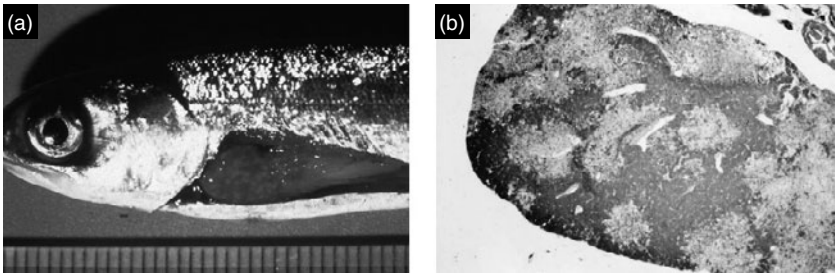


Fig. 7.6. Chum salmon fry exposed to OMV. (a) White spot lesions were observed; (b) multiple foci of severe necrosis in a liver section were observed by HE stain.

specimens from this group. Necrosis of the kidney haematopoietic tissue was observed in infected 3-month-old masu salmon. While the kidney was considered to be the early target organ for OMV, it gradually became resistant to OMV infection. For this reason, it was considered that the principal target organ moved from the kidney to the liver and marked histopathological changes were observed in the later stages. Foci of necrosis in the liver tended to become more severe with longer incubation periods (Fig. 7.6b). Hepatocytes showing margination of chromatin were present. Cell degeneration in the spleen, pancreas, cardiac muscle and brain was also observed (Yoshimizu *et al.*, 1988a). Histopathological changes observed in coho salmon and chum salmon were the same as those of masu salmon (Kumagai *et al.*, 1994). In the case of rainbow trout, high infectivity titres were demonstrated in the main internal organs and multiple necrotic foci were observed in the liver. The definite change was necrosis of OMV infected cells, which were observed in the spleen, haematopoietic tissues in the kidney, liver, intestine, heart, gill filaments, epidermis and lateral musculature. In particular, the intestine showed severe necrosis and haemorrhage in the epithelium and underlying tissues, which is the new description of rainbow trout OMVD (Furihata *et al.*, 2004).

The ultrastructure of RTG-2 cells and hepatocytes of chum salmon infected with OMV was observed by Tanaka *et al.* (1987). Cells were fixed 3 and 7 days after OMV infection and the tissue samples from the liver of moribund 80-day-old, 3-month-old and 4-month-old chum salmon infected with OMV by the immersion method were observed by thin sectioning prepared for electron microscopy. Electron micrographs of both RTG-2 cells and hepatocytes infected with OMV revealed the following activities: nuclei showed margination and the subsequent aggregation of chromatin; syncytia were found in both RTG-2 cells and hepatocytes; the nuclear membranes became smooth and the virus matured in the nucleus of those cells; the matured virus particles were observed in the vesicle and around the cell membrane. The size of enveloped virus particles was approximately 200×240 nm and the nucleocapsid was 115 nm in diameter (Kimura *et al.*, 1981b,c; Tanaka *et al.*, 1987).

Immunity

In masu salmon and chum salmon, following the septicaemia phase of OMV infection, an immune response takes place that results in the synthesis of neutralizing antibodies and ELISA antibody to OMV. In coho salmon, 1-year-old infected fish in particular show ulcers on the skin, white spots on the liver and neoplastic tissues around the mouthparts or body surface; neutralizing antibodies against OMV were detected. Sera of surviving fish, with or without tumours, showed a higher neutralizing antibody titre than the uninfected control fish (Kimura *et al.*, 1981b,c).

Tumour induction

The initial infection by OMV appears as a systemic and frequently lethal infection that is associated with oedema and haemorrhage. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes was recognized. Fifty-two 5-month-old chum salmon that survived experimental infection were held for additional observation. Four months after this first clinical condition, a varying number of surviving fish exhibited epithelioma occurring mainly around the mouth, upper and lower jaw (Fig. 7.7) and subsequently on the eye, under the gill cover and on the caudal fin. Tumours were present in more than 60% of the fish held for 8 months post-infection. Some fish had tumours in multiple sites and one of the 52 fish had a tumour in the kidney. The incidence of tumours on different sites is shown in Fig. 7.8. Tumours in coho and chum salmon were first observed 120 days post-infection and, after 200 days, 35% of the coho and

40–60% of the chum salmon were affected. The rate of tumour induction was not influenced by the age of the fish at the time of infection. Tumours of rainbow trout and masu salmon were not present at 200 days post-infection but appeared after 240 and 270 days, and the rate of tumour inducement reached 12% for rainbow trout and almost 100% for masu salmon after 365 days (Fig. 7.9). Tumours occurred mainly around the mouth, but were also observed on the fins, opercula, body surfaces and cornea. Histopathologically, the tumours were composed of abundantly proliferative, well-differentiated epithelial cells supported by fine connective tissue stroma. OMV was recovered from the culture medium of one passage of the transplanted tumour cells by primary culture series (Yoshimizu *et al.*, 1987).

The perioral site was recognized as the most frequent area for tumour development. Because control fish held under the same conditions showed no tumours, OMV was presumed to have a causal role in tumour development. This neoplasia may persist for up to 1 year post-infection.

Histopathology of tumour tissues

The histological appearance of one oral tumour is shown in Fig. 7.7b. The tumour

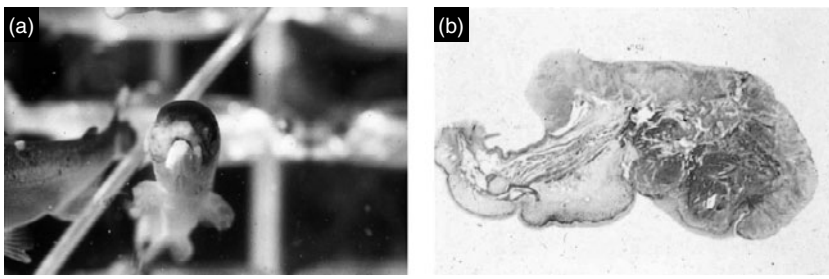


Fig. 7.7. Tumour developing around the mouth of a chum salmon fingerling (a) and histopathological section of tumour tissue from the jaw of the chum salmon (b).

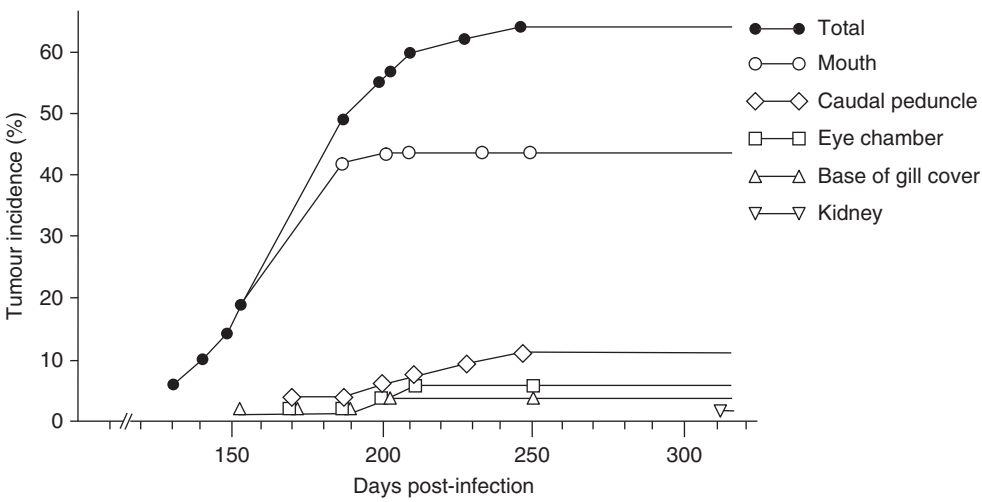


Fig. 7.8. Incidence of tumour development among chum salmon fry following exposure to OMV.

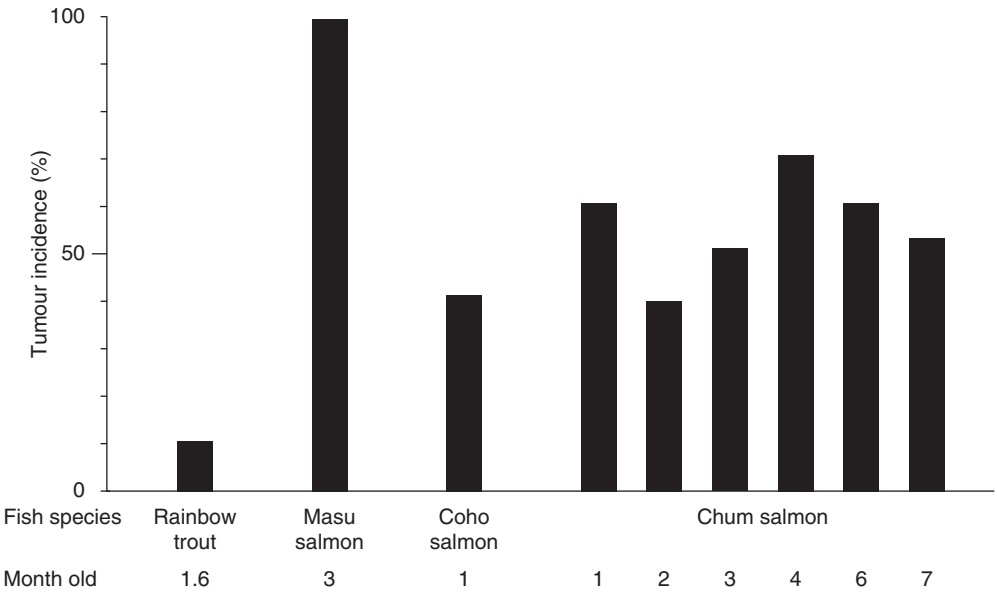


Fig. 7.9. Tumour-inducing rate of four salmonid fish: rainbow trout, chum, masu and coho salmon fry.

cells appear to be epithelial and abundant mitoses suggest a highly proliferative nature. There are several layers of epithelial cells in a papillomatous array and supported by fine connective tissue stroma. Tumours appearing on the caudal fin, gill cover, body surface, corneas of the eye and kidney showed characteristics similar to those of the mouth (Kimura *et al.*, 1981b,c). The tumour formed in the kidney, as well as those from other sites such as on the eye, under the gill cover and caudal fin, showed a similar histopathological pattern.

Electron microscopy revealed that the tumour cells had a typical neoplastic feature of variability in nuclear size and loose intracellular connection. However, OMV particles have not been found in the nuclei or the cytoplasm of the tumour cells (Kimura *et al.*, 1981b,c; Yoshimizu *et al.*, 1987). The virus was isolated from eroded tumour tissue of one fish 9 months after infection and from a primary culture of tumour cells from another fish 10 months after infection. The primary cultures had continuous growth for the first 4 days, which then manifested into CPE-like changes (Fig. 7.10). At this time, OMV was isolated from the culture medium. Neutralizing antibody against OMV was detected among individual and pooled sera from tumour-bearing fish.

Observations were repeated during 1980 using a different batch of chum salmon. Tumours developed at about 4 months post-infection, the same period found in the first experiment. Similar results were observed using coho salmon and rainbow trout. The results suggest that OMV shows pathogenicity for salmonids not only in causing hepatic necrosis but also in inducing tumours among some survivors. In rainbow trout, the diseased fish exhibit almost no external signs, although some fish manifest ulcerative lesions on the skin. Internally, intestinal haemorrhage and white spots on the liver are observed.

Control, Treatment and Epizootiology

Epizootiology

Beginning in the 1980s, OMV was distributed widely in the northern part of Japan. In 1988, OMVD was diagnosed in net pen-reared coho

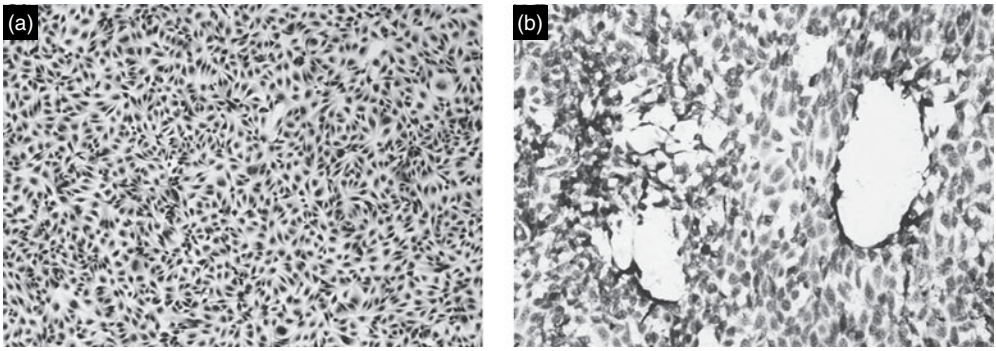


Fig. 7.10. Cytopathic effects produced by OMV. (a) Primary culture cells from tumour tissue; (b) one-time subcultured cells.

salmon in the marine environment in Tohoku District, Japan. OMVD of coho salmon was controlled successfully by Kumagai *et al.* (1994). Since 1991, OMVD has been found in rainbow trout. Recently, OMVD has become a major problem in pond culture of rainbow trout in central Japan. Observations from naturally occurring disease and experimental infections indicate that fish up to 1–5 months of age are most susceptible. In recent years, epizootics have been limited and reported in juvenile and yearling to large rainbow trout (100–500 g), and large-scale loss is reported. Occasionally, mortality may exceed 80%. Most OMVD occurs at 15°C or lower in fresh water. Horizontal transmission has been demonstrated and waterborne transmission can be accomplished in the field. Clinically infected juvenile rainbow trout and carrier adults are the reservoir of virus for waterborne transmission. No other reservoirs of virus have been identified.

Virucidal effects of disinfectants and antiviral chemotherapy

Virucidal effects of six kinds of disinfectants were examined against OMV and infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosis virus (IHNV) and hirame rhabdovirus (HIRRV). At 15°C for 20 min, minimum concentrations showing 100% plaque reduction of OMV by iodophore, sodium hypochlorite solution, benzalkonium chloride solution, saponated cresol solution, formaldehyde solution and potassium permanganate solution were 40, 50, 100, 3500 and 16 mg/l, respectively. Susceptibility of OMV against these disinfectants is higher than that of IPNV, IHNV and HIRRV, suggesting that the usage of disinfectants against IPNV and/or IHNV is also effective against OMV (Hatori *et al.*, 2003).

Minimum inhibitory concentrations (MIC) of BVdU, the highly potent and selective antiherpesvirus agent, (*E*)-5-(2-bromovinyl)-2 deoxyuridine for OMV and *H. salmonis* were 1.25 and 3.0 μg/ml, respectively; these values were equal to or higher than those obtained for ACV (Acyclovir; 9-(2-hydroxyethoxymethyl) guanine). OMV DNA polymerase activity was reduced in a dose-dependent fashion by BVdU 5′-triphosphate (BVdU-TP) within the concentration range of 3–30 μm. However, BVdU-TP could also be substituted for the natural substrate, TTP, in the OMV DNA polymerase assay. It is postulated that the inhibitory action of BVdU on the salmonid herpesviruses is more or less similar to that on other herpesviruses and consists of the inhibition of the virus DNA polymerase activity as well as incorporation of BVdU into the viral DNA (Kimura *et al.*, 1988).

ACV showed high efficacy against the fish herpesvirus OMV, *Herpesvirus salmonis* and channel catfish virus (CCV). Cytopathic effect (CPE) induced by 100 $TCID_{50}$/ml of

OMV in RTG-2 cells was inhibited by 2.5 μg/ml of ACV. ACV was more effective than other compounds such as 9-β-D-arabinofuranosyladenine (Ara-A), 5-iodo-2′-deoxyuridine (IUdR) and phosphonoacetate (PA). Growth of RTG-2 cells was inhibited considerably by ACV at 25 μg/ml, but no morphological changes were observed in the cells. Replication of OMV in RTG-2 cells inoculated with 100 $TCID_{50}$/ml was suppressed completely by 2.5 μg/ml of ACV. Addition of ACV within 4 days post-infection was effective in reducing OMV replication. In order to be effective, ACV had to be present continuously (Kimura *et al.*, 1983a). The therapeutic efficacy of ACV was evaluated using OMV and chum salmon fry. The fish were infected experimentally with OMV and were treated with ACV either orally or by immersion. Daily immersion of fish into ACV solution (25 μg/ml, 30 min/day, 15 times) reduced mortality of the infected fish (Fig. 7.11). Oral administration of the drug (25 μg/fish/day, 60 times) did not affect survival of the chum salmon. On the contrary, the group administered IUdR by the oral route showed a higher survival than the ACV-administered group. This suggested that an effective level of ACV was not maintained in fish given the drug by the oral route. Daily immersion of infected fish into ACV solution (25 μg/ml, 30 min/day, 60 times) suppressed considerably the development of tumours induced by OMV (Kimura *et al.*, 1983b).

Control strategy

OMV is sensitive to ultraviolet irradiation, ozone or iodophore treatment (Kimura and Yoshimizu, 1989; Yoshimizu, 2002, 2009). Since 1983, inspection of the ovarian fluid

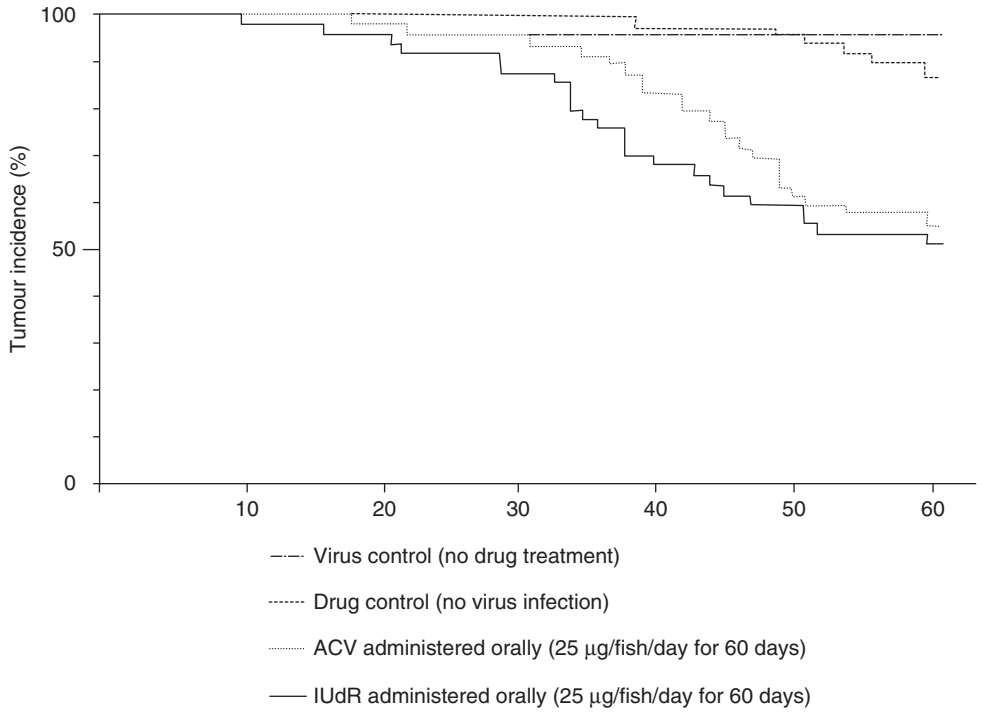


Fig. 7.11. Effect of oral administration of ACV and IUdR on survival of 4-month-old chum salmon fingerlings (body weight 0.69 g) following infection with OMV. OMV inoculation was 100 $TCID_{50}$/ml.

from mature fish and disinfection with iodine of collected eggs at the early eyed stage has been strongly recommended as a control strategy in all hatcheries in Hokkaido. Currently, OMV is no longer detected in most of the hatcheries in this area. Nowadays, all eggs and facilities are disinfected by iodophore just after fertilization and again at the early eyed stage. Vaccination of mature rainbow trout with formalin-inactivated OMV could reduce the positive ratio of OMV in ovarian fluid (Fig. 7.12). Also, vaccination using formalin-inactivated OMV is very effective in protecting OMV infection at the fry stage (Fig. 7.13). As a result, OMV cannot be isolated in most of the hatcheries in this area and the outbreak of OMVD can be avoided (Yoshimizu *et al.*, 1993; Kasai *et al.*, 2004; Yoshimizu, 2009).

General sanitation measures are standard practice in hatcheries (Yoshimizu, 2003). Special care must be taken to avoid

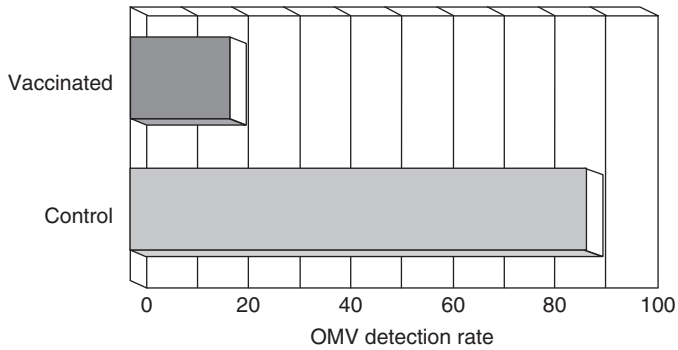


Fig. 7.12. Detection of OMV from ovarian fluid of rainbow trout by PCR. Fish were vaccinated with formalin-inactivated OMV OO-7812 at a dose of $10^{5.0}$ $TCID_{50}$/0.1 ml/fish.

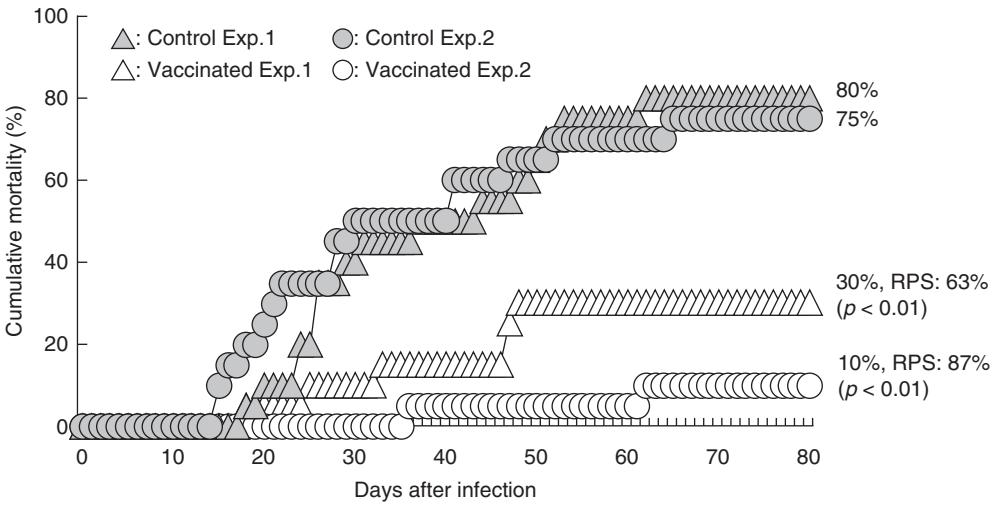


Fig. 7.13. Cumulative mortality of rainbow trout that were injected intraperitoneally with OMV. Fish were vaccinated with formalin-inactivated OMV RtNa-0010 at a dose of $10^{6.7}$ $TCID_{50}$/0.1 ml/fish. Vaccinated or control fish were challenged with $10^{0.9}$ $TCID_{50}$/0.1 ml/fish. No mortality was observed in the control group (not shown). $p < 0.01$ (Fisher's exact probability test).

the movement of equipment from one tank to another and all should be disinfected after use. Methods to sanitize a hatching unit should be developed carefully with respect to chemical toxicity to fish, effects of water temperature and their repeated use. It should be remembered that workers themselves might serve as efficient vectors for pathogens and proper disinfection of hands and boots is required to prevent dissemination of viruses. Although it may be difficult to sanitize hatching and rearing units during use, raceways and ponds should be disinfected with chlorine before and after use (Yoshimizu, 2009).

Water systems provide an efficient means for the introduction and spread of infectious diseases. Pathogen-free water sources are often essential for success in aquaculture. Water commonly used in hatcheries and which come from rivers or lakes contain fish pathogens. Such open water supplies should not be used without treatment to kill fish pathogens. Fish viruses are divided into two groups, based on the sensitivity to UV. Sensitive types are OMV, IHNV, LCDV and HIRRV (Fig. 7.14). These viruses are inactivated by treatment with 10^4 μW s/cm^2 (ultraviolet [UV] dose) (Yoshimizu *et al.*, 1986).

Effective monitoring and management are very important during the collection of eggs in a hatchery. Since some viruses are transmitted vertically from adult to progeny via contaminated eggs or sperm, disinfection of the surface of fertilized eggs has been proven to be effective in breaking the infection cycle for several viruses, such as herpesvirus and rhabdovirus (Yoshimizu *et al.*, 1989; Yoshimizu, 2009). Health inspections of mature fish are conducted to ensure that fish are free from certain important diseases. Routine inspections and specialized diagnostic techniques are required to ensure specific pathogen-free broodstock. For salmonid fish, ovarian fluid is collected by the method of Yoshimizu *et al.* (1985) and routinely inspected following cell culture methods. Fertilized eggs are disinfected with iodophore 25 mg/l for 20 min or 50 mg/l for 15 min. The region inside the

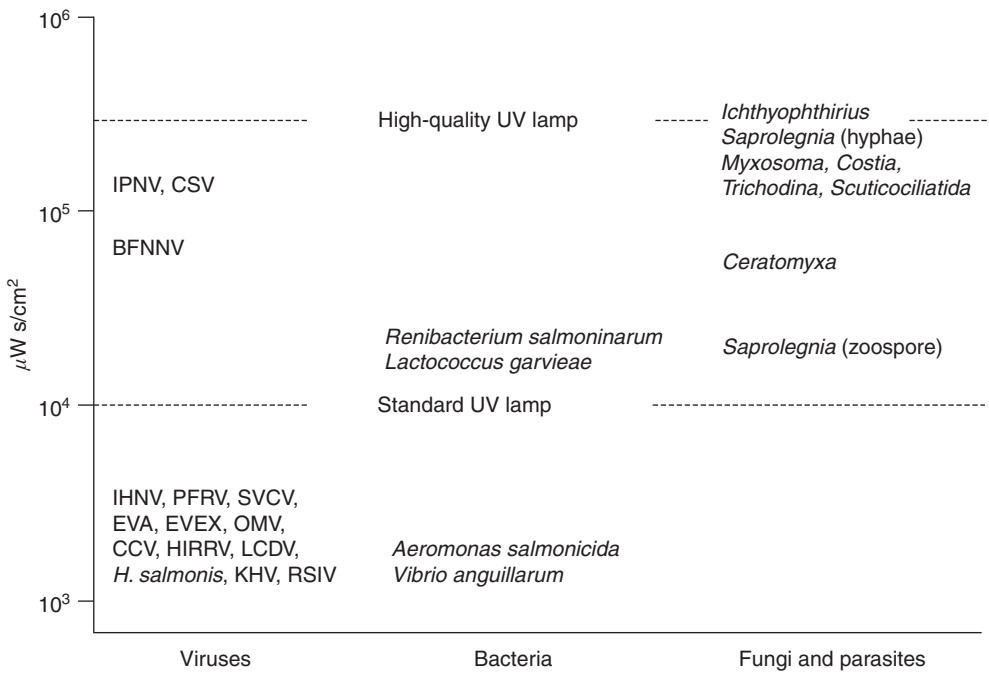


Fig. 7.14. UV susceptibility of fish pathogenic microorganisms (Yoshimizu, 2009).

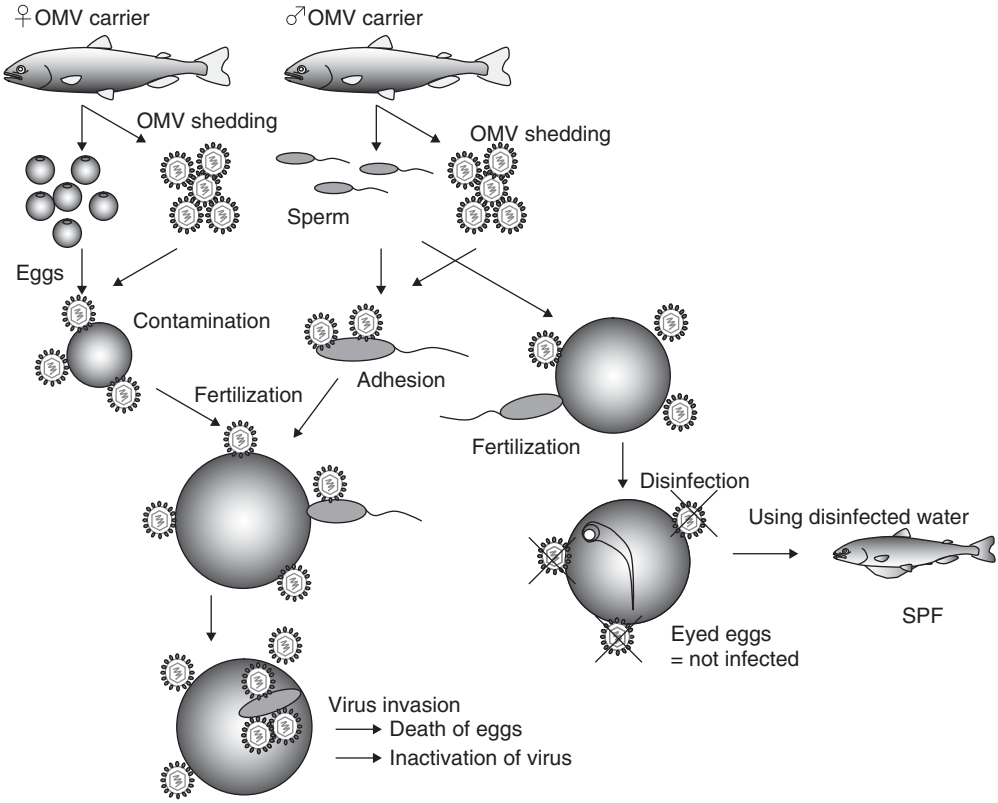


Fig. 7.15. Prevention method of vertical transmission from matured fish to progeny by disinfection of egg surface.

egg membrane of eyed eggs is considered pathogen free (Yoshimizu *et al*., 1989, 2002). It is very important to disinfect the surface of eyed eggs with iodophore (Fig. 7.15).

When fry show abnormal swimming or disease signs, they should be picked up and brought to the laboratory for diagnosis as soon as possible. Moreover, health monitoring should be done regularly. There are many methods for viral detection; generally, cell culture isolation, fluorescent antibody techniques (FAT), immunoperoxidase stain (IPT), antigen-detecting ELISA and PCR tests have been used (Yoshimizu *et al*., 2005).

Acknowledgement

We thank Drs Kerstin Anders and Pornpimol Jearranaiprepame for providing information on fish skin tumours and pike lymphosarcoma.

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8 *Piscirickettsia, Francisella* and Epitheliocystis

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Introduction

Bacteria in the orders *Thiotrichales* (which includes the families *Piscirickettsiaceae* and the *Francisellaceae*) and *Chlamydiales* share a number of characteristics, including intracellular growth, replication by binary fission and a typical Gram-negative cell wall. However, the chlamydiae are distinguished by their unique developmental cycle involving transition from a rigid-walled infectious cell to a flexible replicative form.

A number of species of rickettsiae and chlamydiae are pathogens of humans and other animals. Those best described from fish are the rickettsia that cause piscirickettsiosis (PRS; Fryer *et al.*, 1990) and the chlamydia-like organism (CLO) that causes epitheliocystis (EP; Hoffman *et al.*, 1969). Although the agent that causes EP has never been isolated, the disease has been reported in fish from both warmwater and coldwater habitats and from a variety of hosts, including marine, anadromous and freshwater species. Recognition of the francisellaceae as fish pathogens has occurred only recently (Kamaishi *et al.*, 2005).

The number of bacteria of the *Thiotrichales* recognized in association with disease in fish has increased since 1989, when the first of these agents was isolated from coho salmon (*Oncorhynchus kisutch*) in Chile (Fryer *et al.*, 1990) and subsequently classified as *Piscirickettsia salmonis* (Fryer *et al.*, 1992). Rickettsia-like organisms (RLO) have been observed in a variety of freshwater fish, salt-water fish and invertebrate species (e.g. see Fryer and Lannan, 1994; Mauel and Miller, 2002, and references therein). Piscirickettsia-like organisms (PLO) have been reported from Atlantic salmon (*Salmo salar*) (Corbeil *et al.*, 2005) and several species of tilapia, including *Oreochromis*, *Sarotherodon* and *Tilapia* spp. (Chen *et al.*, 1994; Chern and Chao, 1994; Mauel *et al.*, 2005). Antigenic comparisons of PLO isolates from grouper *Epinephelus melanostigma* in Taiwan (Chen, S.C. *et al.*, 2000) and white sea bass (*Atractoscion nobilis*) in California, USA (Chen, M.F. *et al.*, 2000), confirmed their similarity with *P. salmonis* and later molecular analysis revealed that the white sea bass isolate was identical to *P. salmonis* (Arkush *et al.*, 2005). An isolate from European sea bass (*Dicentrarchus labrax*) was also confirmed as *P. salmonis* using 16S rDNA and internal transcribed spacer sequence comparisons (McCarthy *et al.*, 2005). Other bacteria designated as RLOs or PLOs have greater affinity to the *Francisella*-like bacteria (FLB). Isolates from tilapia from Taiwan and Latin America (Hseih *et al.*, 2007; Mauel *et al.*, 2007) were genetically confirmed as *Francisella* sp., and the similarity

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of these infections to earlier outbreaks (Chen *et al.*, 1994; Chern and Chao, 1994; Mauel *et al.*, 2005) suggested these might also result from FLB. However, the relationships among most RLOs, PLOs and FLB have not been fully evaluated. It is likely that the host range and geographic distribution of these intracellular bacteria will expand as these and possibly other, as yet unknown, isolates are subjected to molecular and antigenic analysis.

Piscirickettsiosis

The disease agent

Aetiological agent

P. salmonis gen. nov., sp. nov. (Fryer *et al.*, 1992) is a Gram-negative, non-motile, intracellular bacterium placed recently in the Class Gammaproteobacteria, Order *Thiotrichales*, Family *Piscirickettsiaceae* (Boone and Castenholz, 2001). This classification was asserted based on the nucleotide sequence and secondary structure of the 16S rDNA gene (Fryer, 2002). Other members of the Gammaproteobacteria include *Coxiella*, *Francisella* and *Legionella*. The *Piscirickettsiaceae* contains five genera, *Cycloclasticus*, *Hydrogenovibrio*, *Methylophaga*, *Piscirickettsia* and *Thiomicrospira*. The genus *Piscirickettsia* contains only one species. The type strain, LF-89, was isolated from a diseased coho salmon collected during an epizootic at a seawater net-pen facility in southern Chile (Fryer *et al.*, 1990). Since that initial isolation, however, *P. salmonis* has been recovered from other salmonid species in both marine (Fryer *et al.*, 1990) and fresh water (Gaggero *et al.*, 1995; Almendras *et al.*, 1997a), as well as two marine fish species (Arkush *et al.* 2005; McCarthy *et al.*, 2005).

The bacterium, *P. salmonis*, is non-motile, pleomorphic but predominantly coccoid and may occur in pairs of curved rods or rings (Fryer *et al.*, 1990). Diameter of individual cells is 0.5–1.5 µm (Fig. 8.1). *P. salmonis* replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of infected fish and in certain cultured fish cell lines (Figs 8.2 and 8.3).

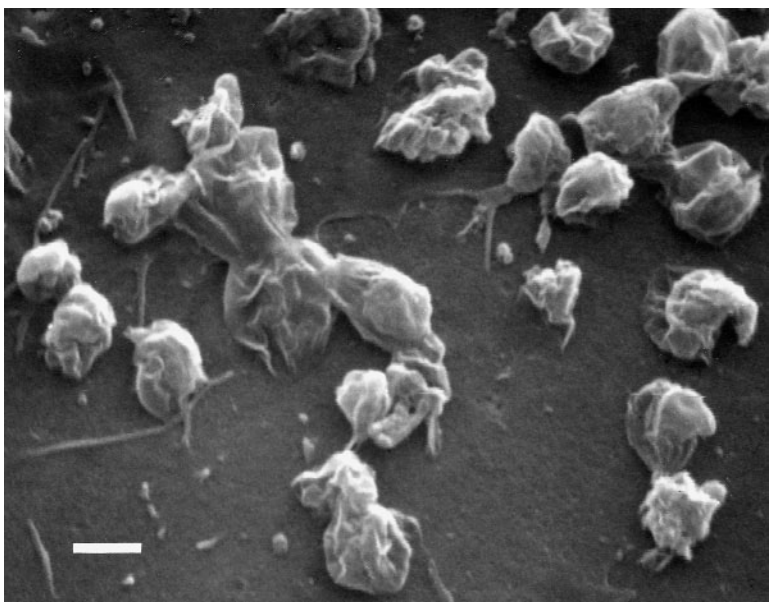


Fig. 8.1. Scanning electron micrograph of *Piscirickettsia salmonis* released from cultured CHSE-214 cells. Rickettsiae of varied sizes are each enclosed in a highly rippled outer membrane. Scale bar, 1 µm.

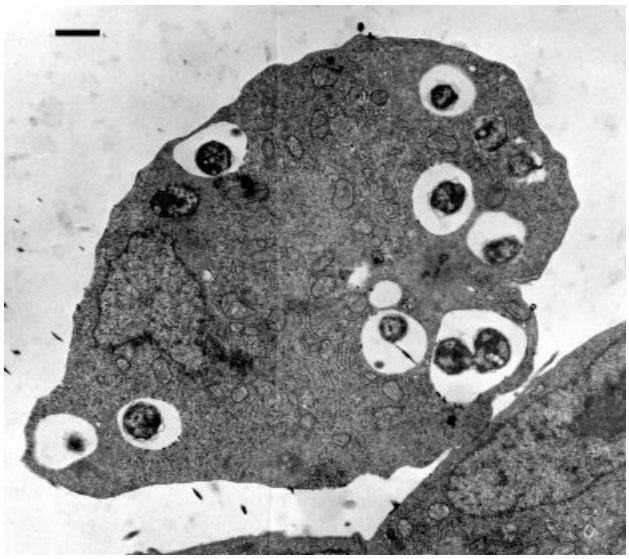


Fig. 8.2. Transmission electron micrograph of *Piscirickettsia salmonis*, replicating within cytoplasmic vacuoles in a CHSE-214 cell. Scale bar, 1 µm.

The bacterium can also be isolated on agar and broth media (Mikalsen *et al.*, 2008). Observations by electron microscopy show the cytoplasm is enclosed by three layers: a rippled outer membrane, a typical Gram-negative cell wall and an inner membrane (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991; Comps *et al.*, 1996; Fryer and Lannan, 1996). The bacterium stains dark blue with Giemsa reagents, is Gimenez-negative (Gimenez, 1964) and retains basic fuchsin when stained by Pinkerton's method for rickettsia and chlamydia (US Surgeon-General's Office, 1944) (Fryer *et al.*, 1990). The type strain (LF-89) has been deposited with the American Type Culture Collection as strain ATCC VR 1361 (Fryer *et al.*, 1992).

Rickettsial isolates from fish in Canada, Chile, Ireland, Norway, Scotland, the USA and the Mediterranean (Table 8.1) are morphologically similar and have been identified as *P. salmonis* using a combination of genetic, biochemical and serological techniques (Lannan *et al.*, 1991; Alday-Sanz *et al.*, 1994). Variation in cytopathic effect in CHSE-214 cells and in antibiotic sensitivity has been reported (Smith *et al.*, 1996b).

Antigenic differences have been reported among suspected *P. salmonis* isolates from salmonid and non-salmonid hosts (Grant *et al.*, 1996; Chen, M.F. *et al.*, 2000). These differences may be explained in part by the use of polyclonal antibodies to *P. salmonis* (LF-89), which may be raised in different species in different laboratories. Several antigens of *P. salmonis* have been identified using both polyclonal and monoclonal antibodies. Rabbit anti-*P. salmonis* (strain LF-89) serum recognized four protein and two carbohydrate antigens with relative molecular weights of 65, 60, 54, 51, 16 and ~11 kDa, respectively (Kuzyk *et al.*, 1996). Sera from convalescent rainbow trout (*O. mykiss*) and coho salmon reacted with protein antigens ranging in size from 10 to 70 kDa and a carbohydrate antigen of ~11 kDa. Kuzyk *et al.* (1996) hypothesized that the 11 kDa carbohydrate antigen was most likely the lipo-oligosaccharide component of the lipopolysaccharide (LPS), which is a component of the outer lipid leaflet of outer membranes of rickettsiae and other Gram-negative bacteria (Amano *et al.*, 1993). Jones *et al.* (1998) described a monoclonal

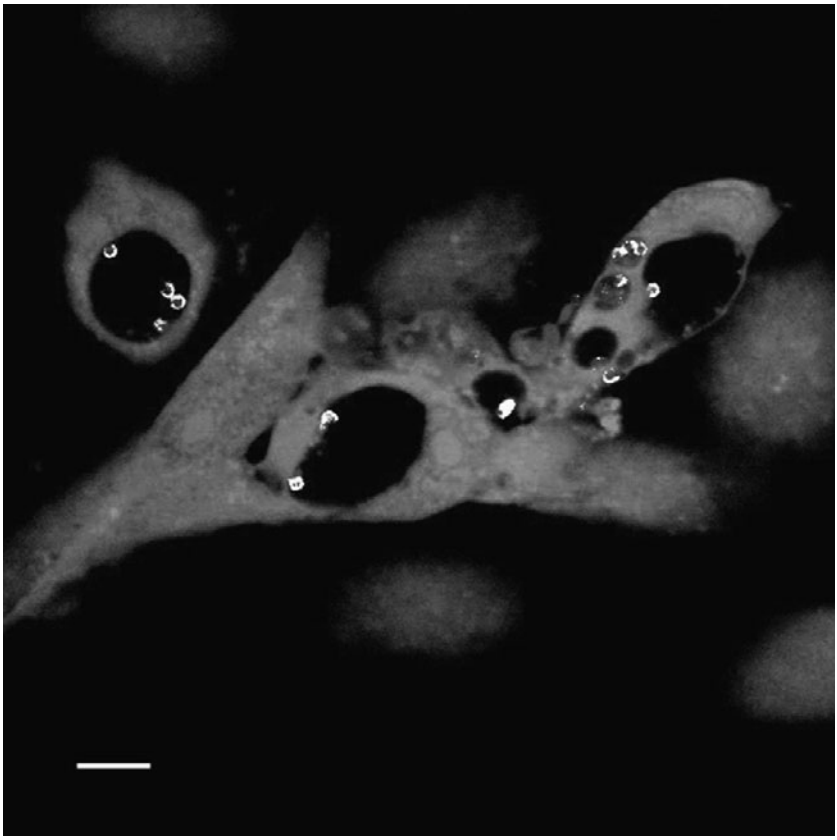


Fig. 8.3. Confocal image of *Piscirickettsia salmonis* in cytoplasmic vacuoles in CHSE-214 cells. Scale bar, 5 µm.

antibody that also recognized a lipopolysaccharide-associated antigen in four RLO isolates isolated from farmed Atlantic salmon in eastern Canada. Barnes *et al.* (1998) showed that rabbit polyclonal antisera recognized three major and five minor antigens of purified LF-89. Variation in electrophoretic profiles of *P. salmonis* passaged in cell cultures has been reported, but only with the type strain LF-89; no comparisons among different strains or geographical isolates have been conducted (Larenas *et al.*, 2006).

Transmission

The source and reservoir of *P. salmonis* in the natural environment is unknown. In terrestrial environments, an intermediate arthropod host or vector is required for transmission of rickettsiae (Weiss and Moulder, 1984) and ectoparasites might serve as potential vectors of *P. salmonis* transmission (Lannan and Fryer, 1994; Fryer and Hedrick, 2003). Using an indirect immunofluorescent test (IFAT), Garcés *et al.* (1994) presented evidence for the presence of the bacterium in the parasitic isopod *Ceratothoa gaudichaudii*, but not in *Caligus* sp. However, a vector is not required for *P. salmonis*; experimental studies have shown that horizontal transmission can occur in both fresh water and seawater (Cvitanich *et al.*, 1991; Almendras *et al.*, 1997a; Smith *et al.*, 1999, 2004) and vertical transmission can occur in fresh water (Larenas *et al.*, 1996, 2003). Smith *et al.* (1999) showed that

Table 8.1. *Piscirickettsia salmonis* observed in and/or isolated from fish.

Fish host species	Geographical location	Salt/ fresh water	Observed/ isolated	Reference
Coho salmon (*Oncorhynchus kisutch*)	Chile	S	I	Fryer *et al*. (1990)
Rainbow trout (*Oncorhynchus mykiss*)	Chile	S	O/I	Garcés *et al*. (1991)
Chinook salmon (*Oncorhynchus tshawytscha*)				
Atlantic salmon (*Salmo salar*)				
Atlantic salmon	Ireland	S	O	Rodger and Drinan (1993)
Masou salmon (*Oncorhynchus masou*)	Chile	S	O	Bravo (1994)
Coho salmon	Chile	F	I	Gaggero *et al*. (1995)
Rainbow trout				
Atlantic salmon	Atlantic coast of Canada	S	O	Cusack *et al*. (2002)
Atlantic salmon	Scotland	S	I	Reid *et al*. (2004)
Atlantic salmon	Norway	S	O/I	Olsen *et al*. (1997)
Pink salmon (*Oncorhynchus gorbuscha*)	British Columbia, Canada	S	O/I	Evelyn *et al*. (1998)
Chinook salmon				
Coho salmon				
Atlantic salmon				
White sea bass (*Atractoscion nobilis*)	Southern California, USA	S	I	Chen, M.F. *et al*. (2000) Arkush *et al*. (2005)
European sea bass (*Dicentrarchus labrax*)	Mediterranean	S	O	Steiropoulos *et al*. (2002) McCarthy *et al*. (2005)

S, host collected from salt water; F, host collected from fresh water; I, isolated in cell culture; O/I, observed and subsequently isolated in cell culture; O, observed only.

the main entry sites of *P. salmonis* in experimentally exposed rainbow trout were the skin and gills, though intestinal intubation and, to a lesser extent, gastric intubation also induced disease. In coho salmon, piscirickettsiosis could be reproduced experimentally by exposure of skin, gills and intestine with *P. salmonis* (Smith *et al*., 2004). In both reports, mortalities following intestinal exposures (originating at either the gastric or anal portions) were considered more difficult to interpret since both were artificial methods and would not be likely under natural conditions. In infected fish, detection of the bacterium in the intestine and kidney tissues along with necrosis and sloughing of mucosal epithelium and renal tubules indicates potential routes of shedding (Cvitanich *et al*., 1991; Arkush *et al*., 2005). Shedding of infected cells from gill filaments and even cannibalism may cause further spread (Arkush *et al*., 2005). Transmission from marine fish hosts to salmonid species by cohabitation

in seawater has been demonstrated (Arkush *et al.*, 2005). Thus, the shedding of the bacterium by infected hosts, survival of the bacterium in seawater and a diverse host population are all factors that facilitate horizontal transmission.

Experimental vertical transmission has been demonstrated by inoculation of male and female rainbow trout broodstock with *P. salmonis* (Larenas *et al.*, 1996, 2003). The bacterium has been detected in the gonad as well as coelomic and seminal fluids of infected fish, and has been observed within the ova of adult fish following experimental inoculation (Larenas *et al.*, 1996). Larenas *et al.* (2003) detected bacteria in the fry when one or both rainbow trout parents were inoculated, though none of the infected offspring showed signs of piscirickettsiosis. In fry created from crosses where one or more parents were inoculated, detection of *P. salmonis* by indirect immunofluorescence varied from 16 to 24% at the alevin stage and from 12 to 16% at the fingerling stage. The bacterium could also be transmitted to ova from non-inoculated females if the eggs were incubated in a medium containing *P. salmonis* during the process of fertilization, resulting in infected progeny. The bacterium was detected in 28% of these fish as fry and 16% as fingerlings. Using scanning electron microscopy, Larenas *et al.* (2003) demonstrated that *P. salmonis* cells could penetrate the interior of the ova through the chorion as early as 1 min after initial contact of the eggs with the bacteria. They also described the development of bacterial membrane extensions, which formed a structure termed 'piscirickettsial attachment complex', enabling attachment of the bacterium to the ovum and linking the rickettsia to each other, forming a matrix. The limited number of naturally occurring piscirickettsiosis cases reported in juvenile fish in fresh water suggests that vertical transmission may be a rare event. No sporogenic stage (resistant to desiccation outside a host or vector, e.g. as seen in *Coxiella burnetti*) has been observed in *P. salmonis*; however, examination of cells for such structures is limited. It has been demonstrated that *P. salmonis* can survive 14 days in seawater (Lannan and Fryer, 1994). In contrast, survival experiments in fresh water reveal an almost immediate deactivation (within seconds) of the pathogen. Thus, rapid attachment of the bacterium and entrance into fertilized ova may be a strategy by which *P. salmonis* can persist. However, horizontal transmission in fresh water is unlikely unless the bacterium is protected by host cell membranes or other biological material.

Geographical distribution and host range

The bacterium is associated with piscirickettsiosis of salmon reared in seawater net pens in Chile, Ireland, Norway, Scotland and the Atlantic and Pacific coasts of Canada (Cvitanich *et al.*, 1991; Brocklebank *et al.*, 1992; Fryer *et al.*, 1992; Rodger and Drinan, 1993; Cusack *et al.*, 2002; Olsen *et al.*, 1997; Evelyn *et al.*, 1998). An RLO observed in farmed Atlantic salmon in south-east Tasmania has been identified provisionally as a PLO, which may expand the geographic distribution of the agent (Corbeil *et al.*, 2005). Coho salmon were originally the principal hosts when *P. salmonis* was first detected in Chile (Garcés *et al.*, 1991). The presence of the bacterium and piscirickettsiosis have now been detected in rainbow trout, Chinook salmon (*O. tshawytscha*), Atlantic salmon, pink salmon (*O. gorbuscha*) and masou salmon (*O. masou*) (Garcés *et al.*, 1991; Bravo, 1994). Two agents characterized originally as *P. salmonis*-like, from white sea bass (*A. nobilis*) (Chen, M.F. *et al.*, 2000) and European sea bass (*D. labrax*) (Comps *et al.*, 1996; Steiropoulos *et al.*, 2002; Athanassoupoulou *et al.*, 2004) have since been confirmed as *P. salmonis*, thus expanding the host range of piscirickettsiosis to include two marine species (Arkush *et al.*, 2005; McCarthy *et al.*, 2005).

It is probable that the condition was first recognized in pink salmon held in seawater for experimental purposes at the Pacific Biological Station in British Columbia, Canada, during the early 1970s (Evelyn *et al.*, 1998). The same disease was observed there intermittently in pink,

Chinook and coho salmon over the next 16 years, with other, co-occurring diseases (Evelyn *et al.*, 1998). *P. salmonis* (ATL-4-91) was isolated in 1992 from diseased Atlantic and Chinook salmon held in seawater net pens in British Columbia (Brocklebank *et al.*, 1992). Now, piscirickettsiosis occurs along the coast of British Columbia, and often co-occurs with bacterial kidney disease, caused by *Renibacterium salmoninarum* (Evelyn *et al.*, 1998). On the east coast of Canada in Nova Scotia, the bacterium has been isolated from net-pen reared Atlantic salmon (Jones *et al.*, 1998; Cusack *et al.*, 2002).

By the mid-1980s, the disease was recognized among cultured salmonids in southern Chile near Puerto Montt (Bravo and Campos, 1989). Originally, it was described in coho salmon only, but as other salmonid species were evaluated as candidates for commercial aquaculture, the agent was soon detected in Atlantic salmon, Chinook salmon, rainbow trout (Cvitanich *et al.*, 1991) and masou salmon (Bravo, 1994). Recurring outbreaks resulted in losses of coho salmon exceeding 90% at numerous production sea farms (Bravo and Campos, 1989; Fryer *et al.*, 1990; Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991). Mixed infections with *R. salmoninarum* in both fresh water and seawater have been reported (Cvitanich *et al.*, 1991; Gaggero *et al.*, 1995; Smith *et al.*, 1995).

In 1992, another isolate of *P. salmonis* (NOR-92) was recovered from Atlantic salmon held in seawater net pens on the west coast of Norway (Olsen *et al.*, 1997). The bacterium was also observed in histological specimens from Atlantic salmon cultured in seawater net pens in the west of Ireland (Rodger and Drinan, 1993). These pathogenic microorganisms subsequently were identified as *P. salmonis* using the fluorescent antibody test (Fryer and Lannan, 1996). Analysis of the 16S rRNA gene sequence of the isolates from Norway, British Columbia (Mauel *et al.*, 1999), Scotland and Ireland (Reid *et al.*, 2004) has confirmed their identity. With confirmation of *P. salmonis* as the causative agent of mortality in white sea bass in southern California and European sea bass in the Mediterranean, the agent now appears to have a global distribution, occurring in both marine and freshwater fish.

Economic importance of the disease

Since 1980, salmonid aquaculture has grown quickly into an important industry of Chile, and while no serious diseases were reported in the early years, in 1995 over 10 million coho salmon died during the seawater rearing phase, and most of that mortality was attributed to piscirickettsiosis. Economic loss was estimated at US$49 million in that year alone (Smith *et al.*, 1997). Annual losses due to piscirickettsiosis have had an important effect on the economy in Chile, a country that is second to Norway as the largest exporter of salmon and trout (Wilhelm *et al.*, 2006). However, severity of the disease is reduced today, presumably due to a shift from rearing coho salmon to the more resistant Atlantic salmon, as well as improved prevention and control methods (Fryer and Hedrick, 2003).

Diagnostic methods

Presumptive diagnosis of *P. salmonis* is based largely on microscopic observations of stained tissue imprints or smears or isolation of the bacterium by cell culture (Fryer *et al.*, 1990). Confirmation of *P. salmonis* is made typically by indirect fluorescent antibody test (Lannan *et al.*, 1991; OIE, 2006), immunohistochemistry (Alday-Sanz *et al.*, 1994; OIE, 2006) or polymerase chain reaction assay (Mauel *et al.*, 1996; Marshall *et al.*, 1998; House and Fryer, 2002; OIE, 2006).

P. salmonis may be isolated from infected fish by co-cultivation of infected tissues (e.g. kidney, liver and blood) on a susceptible cell line. The agent has been shown to replicate in a number of fish cell lines, including Chinook salmon embryo (CHSE-214; ATTC CRL 1681; Lannan *et al.*,

1984), chum salmon heart (CHH-1; ATCC CRL 1680; Lannan *et al.*, 1984), coho salmon embryo (CSE-119; Lannan *et al.*, 1984), rainbow trout gonad (RTG-2; ATCC CCL 55; Wolf and Quimby, 1962), *epithelioma papulosum cyprini* (EPC; Fijan *et al.*, 1983) and fathead minnow (FHM; ATCC CCL 42; Gravell and Malsburger, 1965) (Fryer *et al.*, 1990). Almendras *et al.* (1997b) showed that *P. salmonis* could replicate in brown bullhead catfish cells, but that CPE did not appear until 45 days post-inoculation. Birkbeck *et al.* (2004a) demonstrated approximately 100-times greater bacterial cell yields in the Sf21 insect cells (from the fall armyworm, *Spodoptera frugiperda*) compared with CHSE-214 cells, with a maximum temperature for growth in the Sf21 cells of 24°C. Because the organism is sensitive to most of the antibiotics used routinely in cell culture (Lannan and Fryer, 1991), all media, including tissue transport or processing buffers, should be antibiotic free. The tissue is removed aseptically and kept at 4°C, as the bacterium is sensitive both to elevated temperatures and to freezing. Inoculated cultures are incubated at the optimal growth temperature of 15–18°C and observed up to 28 days for appearance of cytopathic effect (CPE) (Fryer *et al.*, 1990). Characteristic CPE appears as plaque-like clusters of rounded cells with large vacuoles. At primary isolation, CPE may require 21 or more days to appear. In subsequent passages, CPE becomes apparent 4–7 days after inoculation. The organism can produce a titre of 10^6–10^7 50% tissue culture infective doses/ml in cultured fish cells. *In vitro* replication is inhibited greatly at temperatures above 20°C and below 10°C, and does not occur at or above 25°C (Fryer *et al.*, 1990). *P. salmonis* is sensitive to freezing and titres may be decreased by 99% with a single cycle of freeze–thaw at –70°C. However, addition of 10% dimethylsulfoxide to the freezing medium as a cryoprotectant improves survival tenfold over cultures frozen without additives (Fryer *et al.*, 1990).

Although isolation in cell culture provides a sensitive method for detection, there are disadvantages. Conditions in the field are rarely ideal for an aseptic necropsy and the necessary absence of antibiotics during subsequent culture steps can result in the introduction of contaminating organisms. Infection with *Mycoplasma* spp. can also pose a problem for antibiotic-free cell cultures (Kuzyk *et al.*, 1999; Thornton *et al.*, 1999). Recently, however, *P. salmonis* was cultured successfully on agar and in broth containing enhanced levels of cysteine, with optimal growth observed at 22°C (Mikalsen *et al.*, 2008). The media, cysteine heart agar blood (CHAB), consists of 25 g/l Bacto heart infusion broth supplemented with 10 g/l glucose, 1 g/l L-cysteine, 15 g/l agar, 2 g/l haemoglobin and 5% ovine blood. Cysteine is a common requirement for other intracellular bacteria, though the authors report limited growth of *P. salmonis* when subcultured on to blood agar without cysteine supplementation. Optimal growth on CHAB was obtained at 22°C and at 4–5 days incubation, the approximately 1 mm diameter colonies were convex, grey-white, shiny, centrally opaque, with translucent, slightly undulating margins. DNA sequencing confirmed that the colonies grown on CHAB were *P. salmonis* and virulence for Atlantic salmon was demonstrated in a laboratory trial. Mauel *et al.* (2008) also reported successful cultivation of *P. salmonis* on a similarly enriched 5% sheep blood agar containing 0.1% cysteine and 1% glucose, designated BCG. BCG plates inoculated with infected CHSE-214 cell culture supernatants and incubated at 16°C for 6 days developed small (0.1 mm) circular, white, convex colonies which could then be reintroduced into CHSE-214 cells and re-establish intracellular infections. Bacteria harvested from both agar and cell culture media were confirmed as *P. salmonis* by molecular detection. This cost-effective, simple isolation method might be suitable for initial identification of the agent and would allow for easier transport of isolates to diagnostic facilities for confirmatory tests. Sensitivity and specificity of the enriched blood agar media for primary isolation from fish tissues versus cell culture methods have not yet been evaluated.

A less sensitive but more rapid method for diagnosis of a rickettsial infection in fish is by microscopic examination of smears or impressions of kidney, liver or spleen tissue stained with Gram, Giemsa or methylene blue solutions (Fryer *et al.*, 1990; Lannan and Fryer, 1991). In Giemsa-stained preparations, the pleiomorphic bacteria will appear darkly stained in coccoid or ring forms, frequently in pairs, with a diameter of 0.5–1.5 µm (Fig. 8.4). These intracellular bacteria may be found in host cell cytoplasmic vacuoles.

Following initial detection in stained tissue smears or impressions, paraffin-embedded tissue sections, agar or broth culture, or cell cultures, identity of *P. salmonis* must be confirmed by means of serological methods, e.g. immunofluorescence, immunohistochemistry or molecular techniques. *P. salmonis* can be confirmed by a fluorescent antibody test (Lannan *et al.*, 1991; Larenas *et al.*, 1996; Jamett *et al.*, 2001). Both polyclonal rabbit anti-*P. salmonis* serum and monoclonal mouse antibodies have been used in the tests described to date. Alday-Sanz *et al.* (1994) described an immunohistochemical assay for detecting the pathogen in infected tissues of Atlantic salmon using rabbit polyclonal anti-*P. salmonis* serum, and a similar test was developed to detect the bacterium in European sea bass (Steiropoulos *et al.*, 2002). An enzyme-linked immunosorbent assay using mouse monoclonal antibodies was described recently (Aguayo *et al.*, 2002) which may avoid variability seen with different polyclonal preparations or cross-reactions with fish or cellular antigens (e.g. Lannan *et al.*, 1991; Barnes *et al.*, 1998). Alternatively, the presence of *P. salmonis* in cell cultures, fish tissue or serum samples can be established via polymerase chain reaction (PCR) assays using primer sets which amplify target sequences in the small subunit ribosomal (16S) gene (Mauel *et al.*, 1996) or the

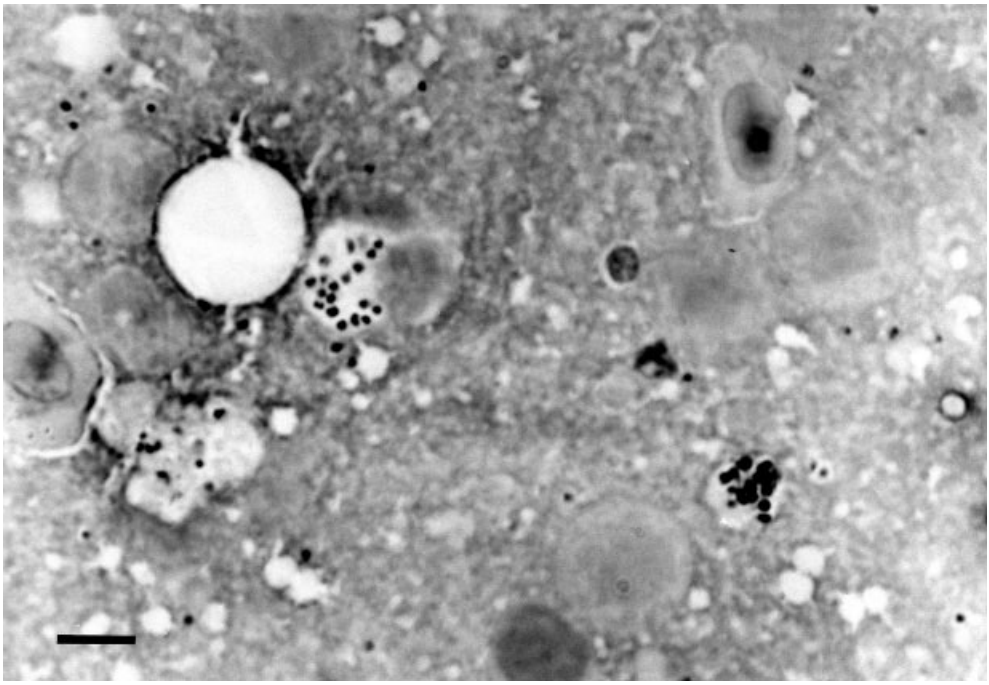


Fig. 8.4. Kidney impression from moribund coho salmon (*Oncorhynchus kisutch*). Note the *Piscirickettsia salmonis* organisms within and scattered among the host cells. Giemsa stain. Scale bar, 10 µm. Photograph courtesy of Sandra Bravo, Puerto Montt, Chile.

internal transcribed spacer (ITS) region between the 23S and the 16S ribosomal subunit genes (Marshall *et al.*, 1998). *P. salmonis* may also be measured quantitatively using a TaqMan PCR test (Corbeil *et al.*, 2003) or with a competitive PCR test (Heath *et al.*, 2000). Following each of these PCR assays, the appropriately sized amplicon may be sequenced for further confirmation.

PCR may also be used to detect *P. salmonis* DNA or genome equivalents from environmental samples. For example, Heath *et al.* (2000) reported the detection of approximately 3000–4000 *P. salmonis* cells (or their DNA remnants) per litre of surface seawater collected from an active net pen during a disease episode, when fish mortality was occurring at a rate of 2.1%/week. Also, *P. salmonis*-like DNA has been detected in bacterioplankton DNA extracted from coastal waters of western USA (Mauel and Fryer, 2001).

Finally, DNA hybridization techniques may be employed as rapid diagnostic and confirmatory tests. A dot blot hybridization test may detect *P. salmonis* from both fish tissues and cell culture supernatants, while *in situ* hybridization (ISH) can be used to visualize infected cells in tissue sections (Venegas *et al.*, 2004). A DNA microarray, by which PCR products or genomic DNA samples can be interrogated for the presence of 15 fish pathogens, including *P. salmonis*, has also been described (Warsen *et al.*, 2004).

Genome structure and transcription

Expression libraries of *P. salmonis* have been constructed (Kuzyk *et al.*, 2001a; Miquel *et al.*, 2003). One report describes a 486 bp open reading frame (ORF), *ospA*, which encodes for a 17 kDa antigenic outer surface lipoprotein (OspA) (Kuzyk *et al.*, 2001a,b). Using a comparative genomics approach, optimal candidates for a recombinant subunit vaccine were identified from a genomic library of *P. salmonis* (Wilhelm *et al.*, 2006). Fifteen *P. salmonis* ORFs encoding heat-shock proteins, virulence factors, membrane-bound and other antigens were isolated and expressed (Wilhelm *et al.*, 2003, 2006).

Comparisons of the 23S and 16S ribosomal RNA genes and the internal transcribed spacer (ITS) regions of several geographically distinct isolates (from Chile, Norway and Canada) of *P. salmonis* have been used to show that they cluster closely into a monophyletic group with one outlier, the Chilean isolate EM-90 (Mauel *et al.*, 1999; Heath *et al.*, 2000). Sequence homologies among isolates of *P. salmonis* from salmon for these ribosomal genes ranged from 95.2 to 99.7%, with similarities ranging from 98.5 to 99.7% for 16S rDNA sequences alone (Mauel *et al.*, 1999). Experimental transmission studies with isolates from Chile (LF-89), Norway (NOR-92) and Canada (ATL-4-91) used in the genetic comparisons by Mauel *et al.* (1999) demonstrated that all were pathogenic to coho salmon, with LF-89 significantly more virulent than the NOR-92 isolate (House *et al.*, 1999). Comparisons including DNA sequences from 16S, 23S and the ITS genes of the salmonid strains show low levels of divergence between isolates but a wider range of similarities than in using 16S rDNA sequences alone (Mauel *et al.*, 1999). Reid *et al.* (2004) analysed 16S–23S ITS sequences and 16S rDNA of a larger set of isolates, including those from Scotland and Ireland, and found that Irish isolates of *P. salmonis* formed unique clusters of the organism. They concluded that two Irish isolates were related to EM-90, while two other Irish isolates formed a new, distinct group more distant from all other recognized isolates. Scottish isolates clustered together with Norwegian and Canadian isolates from Atlantic salmon. Casanova *et al.* (2001) identified two ITS sequences in the type strain, LF-89, and in EM-90, suggesting that more than one *rrn* operon might exist in these Chilean isolates. Two ITS products were confirmed, with a difference in electrophoretic mobility (higher versus lower electrophoretic mobility), in a broader comparison of the ITS amplification products of 11 Chilean *P. salmonis* isolates (Casanova *et al.*, 2003). A higher ITS sequence homology inside each

group was demonstrated by heteroduplex mobility assay (HMA). No association between ITS electrophoretic mobility and geographic origin of the isolates tested was observed, though the authors noted that ITS electrophoretic mobility and host origin appeared to be linked: strains from *Oncorhynchus* differed from those recovered from Atlantic salmon. The authors concluded that PAGE analysis of ITS and HMA could be used for screening genetic variability, and possibly virulence differences, between *P. salmonis* isolates. Alternatively, denaturant gradient gel electrophoresis and constant denaturant gel electrophoresis, which allow separation of PCR amplicons differing by as little as one base change, may be used to analyse strain variation (Heath *et al.*, 2000). In a comparison of isolates LF-89, EM-90, SLGO-94, C1-95, ATL-4-91 and NOR-92, the authors found four migration classes, which corresponded with sequence comparisons. This analytical combination could be useful in monitoring for emerging variants.

Isolates of *P. salmonis* from marine fish species show comparable homologies with those from salmonid fish. A pair-wise comparison between 16S rDNA sequences of the California isolate from white sea bass (WSB-98) and *P. salmonis* strains from Chile, Norway and British Columbia demonstrated homologies of: LF-89 (99.0%), EM-90 (96.6%), NOR-92 (98.5%) and ATL-4-91 (98.2%) (Arkush *et al.*, 2005). PCR amplification of WSB-98, using the diagnostic test described by Mauel *et al.* (1996), produced an amplicon of 467 bp which differed from LF-89 by a total of 4 bp (Arkush *et al.*, 2005). McCarthy *et al.* (2005) reported sequence similarity between the 16S rDNA gene from a *P. salmonis* isolate from the European sea bass (SBPLO) and LF-89 (98.2%), ATL-4-91 (98.7%), Scottish isolates SCO-95A and SCO-02A (99.3%), Irish strains IRE-99D, IRE-98A and IRE-91A (98.0–97.1%) and EM-90 (97.1%). To date, sequence comparisons including 16S, 23S and the ITS region for all of the *P. salmonis* isolates have helped to elucidate their phylogenetic relationships. Ultimately, however, DNA–DNA hybridization studies may be needed for further discrimination among isolates, perhaps resulting in the creation of separate species (McCarthy *et al.*, 2005).

Pathogenesis and immunity

Disease

Infections of *P. salmonis* in salmonid fish have been referred to as 'coho salmon syndrome' or 'Hitra disease' (Branson and Nieto Díaz-Munoz, 1991), 'salmonid rickettsial septicaemia' (Cvitanich *et al.*, 1991) and 'piscirickettsiosis' (Fryer *et al.*, 1992; Lannan and Fryer, 1993), and initially the pathogen was termed 'UA', or unknown agent (Branson and Nieto Díaz-Munoz, 1991).

In salmonid hosts, piscirickettsiosis generally begins 6–12 weeks following the introduction of salmon or rainbow trout to seawater net pens (Bravo and Campos, 1989; Fryer *et al.*, 1990, 1992; Cvitanich *et al.*, 1991). Infections in fresh water have been reported but are rare (Bravo, 1994; Gaggero *et al.*, 1995). A marine reservoir for *P. salmonis* has been suggested since salmon are not native to the southern hemisphere (Donaldson and Joyner, 1983), where the disease was first reported. Indeed, *P. salmonis* isolated from white sea bass has been shown to induce 80% mortality within 10 days of intraperitoneal injection into juvenile coho salmon (Chen, M.F. *et al.*, 2000), with a virulence equal to or greater than the type strain, LF-89 (House *et al.*, 2006). In addition, the bacterium rapidly loses viability in fresh water (Lannan and Fryer, 1994) where egg incubation and early salmon rearing occur. However, vertical transmission has been shown to occur when either or both parental broodstock are infected experimentally (Larenas *et al.*, 1996, 2003).

External signs of the disease are variable but include inappetence and darkened body coloration, and affected fish may be lethargic and swim near the water surface or at the perimeter of the enclosures (Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991; Brocklebank *et al.*, 1992; Olsen *et al.*, 1997; Evelyn *et al.*, 1998). Erratic swimming behaviour has been noted in

infected coho salmon and, in these cases, the bacterium was isolated from the brain of symptomatic fish (Skarmeta *et al.*, 2000). Similarly, abnormal swimming and whirling behaviour were exhibited by infected juvenile sea bass (*D. labrax*) (McCarthy *et al.*, 2005). Infected fish may have skin lesions that range from small areas of raised scales to shallow haemorrhagic ulcers (Branson and Nieto Díaz-Munoz, 1991). Anaemia, which may be evident by pale gills, is a consistent finding. Haematocrits as low as 2% have been reported (Branson and Nieto Díaz-Munoz, 1991), with an average value of 18.5% in infected fish as compared with 35–50% in apparently healthy animals (Cvitanich *et al.*, 1991). However, clinical signs and external lesions may be absent in affected fish, and mortality may occur without gross signs of disease (Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991; Arkush *et al.*, 2005).

Internally, the rickettsiae spread systemically, resulting in swollen kidney and spleen. Although not common in infected fish (5–10%), the most diagnostic lesions occur in the liver as 5–6 mm diameter white or cream-coloured, circular opaque nodules, or as ring-shaped foci (Cvitanich *et al.*, 1991; Lannan and Fryer, 1993; Rodger and Drinan, 1993; Olsen *et al.*, 1997) (Fig. 8.5). Ascites are often present, as are petechial haemorrhages which occur in the body musculature, visceral organs including the stomach, intestines, pyloric caecae and swim bladder, and adipose tissue (Schäfer *et al.*, 1990; Cvitanich *et al.*, 1991; Brocklebank *et al.*, 1993; Rodger and Drinan, 1993). Microscopically, these changes are accompanied by extensive necrosis of haematopoietic tissues. Large numbers of macrophages containing *P. salmonis* cells and cellular debris can be detected in peripheral blood smears. The rickettsia are seen in cytoplasmic vacuoles of cells from the kidney and other organs (Fig. 8.4). Macrophages filled with rickettsial cells are common.

Histopathology associated with natural and experimental infections of *P. salmonis* has been described (Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991; Garcés *et al.*, 1991; Arkush *et al.*, 2005; see also reviews by Almendras and Fuentealba, 1997; Fryer and Hedrick, 2003). Pathological changes occur throughout internal organs including kidney, liver, spleen, heart, brain,

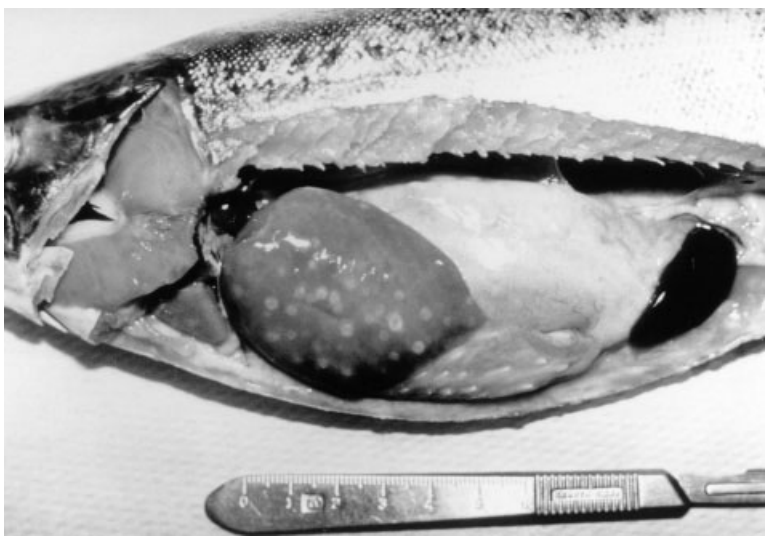


Fig. 8.5. Gross pathology associated with piscirickettsiosis in coho salmon (*Oncorhynchus kisutch*). Note the enlarged spleen, petechiae on internal organs and mottled lesions on the liver. Photograph courtesy of Sandra Bravo, Puerto Montt, Chile.

intestine, ovary and gill, with the most severe manifestations of the disease in the liver, kidney, spleen and intestine (Cubillos *et al.*, 1990; Schäfer *et al.*, 1990; Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991; Larenas *et al.*, 1995; Palmer *et al.*, 1996; Olsen *et al.*, 1997; Chen, M.F. *et al.*, 2000; Arkush *et al.*, 2005). In the kidney and spleen, the normal haematopoietic and lymphoid tissues are replaced with chronic inflammatory cells and host cell debris. Liver lesions, including focal to diffuse necrotizing hepatitis and macrophage aggregates (Fig. 8.6), are severe and rickettsia are often seen in the cytoplasm of degenerating hepatocytes and in macrophages, or free in the blood (Cvitanich *et al.*, 1991; Arkush *et al.*, 2005). Foreign body-type granulomas in liver tissue have been reported (Arkush *et al.*, 2005). In more acute infections, discrete nodules may be absent but rather areas of necrosis coalesce and replace hepatic parenchyma (Arkush *et al.*, 2005). Vascular and perivascular necrosis may be present in the liver, kidney and spleen, with intravascular coagulation resulting in fibrin thrombi within small blood vessels and infiltration by inflammatory cells (Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991). Granulomatous inflammation of the lamina propria and tunica submucosa of the large intestine is common and often results in necrosis and sloughing of the mucosal epithelium. Granulomatous inflammation and hyperplasia of gill epithelia result in fusion of the lamellae, and rickettsia may be found in the secondary lamella blood spaces (Branson and Nieto Díaz-Munoz, 1991). Meningitis, endocarditis and pancreatitis may be present (Brocklebank *et al.*, 1993; Comps *et al.*, 1996; Grant *et al.*, 1996; Skarmeta *et al.*, 2000; Athanassopoulou *et al.*, 2004). Granulomatous inflammation and necrosis of the dermis and epidermis, with degeneration of the subdermal musculature, may be observed (Brocklebank *et al.*, 1993; Chen, M.F. *et al.*, 2000).

Pathogenesis

Although there are no reports of the sequential development of naturally acquired infections with *P. salmonis*, the pathogenesis of this bacterium can be estimated by a combination of reports from both natural and experimentally induced infections. Almendras *et al.* (1997a) demonstrated horizontal transmission of *P. salmonis* in Atlantic salmon by cohabiting experimentally exposed fish with naive fish, either with or without physical contact. Fish were exposed via intraperitoneal injection, oral intubation or application of *P. salmonis* infected cell culture supernatant directly on to the surface of the gills. Both groups that were cohabited with the exposed fish, whether in direct contact or not, became infected. In the directly challenged fish, a capsular (serosal) pattern of *P. salmonis* infection was observed in the liver and spleen of injected fish as early as 7 days post-challenge at 11°C, which later spread to leucocytes and other parenchymal cells of the spleen, liver and kidney. In fish exposed via oral and gill routes, and in both contact and non-contact cohabitants, the infection was characterized by a haematogenous pattern, whereby the bacterium invaded the organs from the blood vessels to the surrounding tissues. Infected leucocytes were found within hepatic sinusoids and blood vessels. Again, infections were detected as early as 7 days post-exposure. Vascular lesions in experimentally infected Atlantic salmon appear to be the result of the tropism of *P. salmonis* for endothelial cells, whereas lesions in organs such as the liver are the result of ischaemic necrosis and direct injury by the intracytoplasmic bacteria (Almendras *et al.*, 2000). In experimental challenges, coho salmon were more susceptible than rainbow trout to piscirickettsiosis (Smith *et al.*, 1996a). Rainbow trout could be infected via patch contact on intact skin, with haemorrhagic lesions developing in the skeletal muscle at the infection site and a cumulative mortality rate of 52% in one study (Smith *et al.*, 1999). These skin lesions were similar to those described in natural infections (Branson and Nieto Díaz-Munoz, 1991). Skin and gills were also effective entry portals for *P. salmonis* in experimentally exposed coho salmon (Smith *et al.*, 2004). Whether by initial serosal or

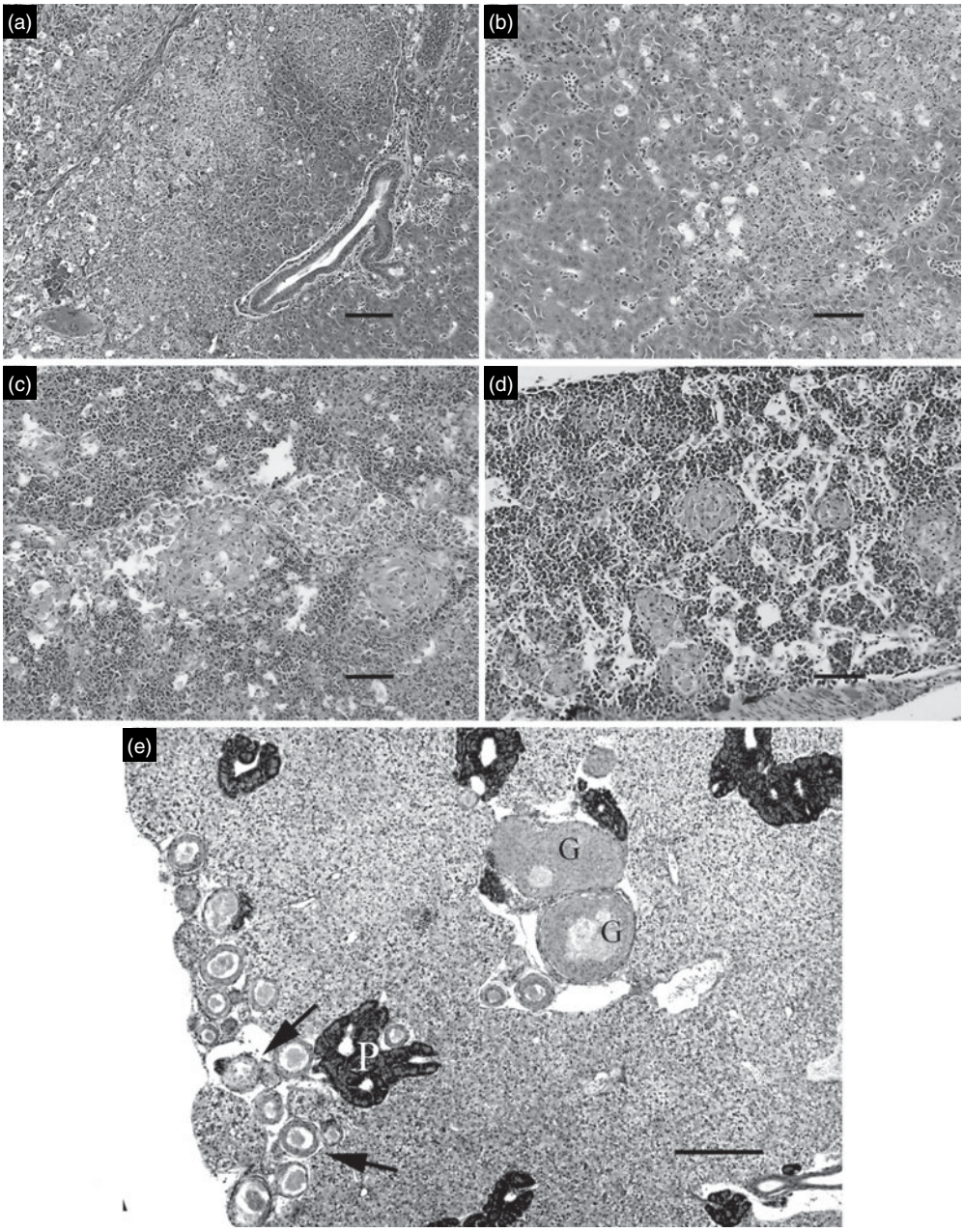


Fig. 8.6. Microscopic lesions in H & E stained tissue sections from white sea bass (*Atractoscion nobilis*) following experimental infections with *Piscirickettsia salmonis*. (a) Focal hepatic lesions characterized by necrosis and infiltration with mononuclear cells. Scale bar = 100 µm. (b) A focus of necrosis in the liver. Scale bar = 50 µm. (c) A focus of necrosis in the spleen. Scale bar = 100 µm. (d) Multiple and focal aggregates of macrophages in the interstitium of the anterior kidney. Scale bar = 100 µm. (e) Large and well-developed granulomas (G) and smaller and numerous granulomas (arrowed) near islet of exocrine pancreas (P) in liver of white sea bass 123 days after intraperitoneal injection with the bacterium. Scale bar = 200 µm. Images in (a), (d) and (e) reprinted with permission.

haematogenous dissemination of the bacterium, later stages of infection are characterized by similar internal gross and microscopic pathological changes in several organs, including liver, spleen, kidney, intestine, heart, gonads and gills, probably because septicaemia ensues (Cvitanich *et al.*, 1991; Almendras *et al.*, 1997a; Arkush *et al.*, 2005). In white sea bass, a histiocytic inflammatory response and organization of macrophages into discrete aggregates facilitate the invasion of blood vessels and spread to other tissues via infected macrophages (Arkush *et al.*, 2005). In the chronic form of the disease, foreign body granuloma formation may allow the persistence of the bacterium in the host. In white sea bass, *P. salmonis* antigens were detected in granulomatous lesions at 4 months post-exposure (Arkush *et al.*, 2006). Survival and persistence of *P. salmonis* in an intracellular location, especially within granulomatous lesions, enables evasion of host immunological surveillance. However, reactivation of these lesions has not been demonstrated experimentally, and thus a true carrier state has not been established.

Virulence factors

As the first isolate of *P. salmonis*, LF-89 is the reference strain (Fryer *et al.*, 1990). Several strains have been described subsequently, with modest sequence differences (Mauel *et al.*, 1996; Reid *et al.*, 2004). By conducting fish exposure studies, Smith *et al.* (1996a) and House *et al.* (1999) have demonstrated variation in strain virulence among isolates from Chile and other countries. However, virulent strains of the bacterium have been shown to lose their pathogenicity in laboratory subcultivation, which may have important implications for studies of the comparative virulence of different host or geographical isolates, or the development of diagnostic tests or effective vaccines. When LF-89 was used to infect coho salmon after the 13th (P13) and 42nd (P42) passages in cell culture, none of the latter exposure group died, while 70% cumulative mortality had occurred in the former group by 30 days post-inoculation (Larenas *et al.*, 2006). By SDS-PAGE, four peptide bands (ranging in size from 70 to 110 kDa) were found in P42 but not in P13, suggesting that electrophoretic patterns of strains may be useful in predicting pathogenicity.

One potential virulence factor of *P. salmonis* is a component of its lipopolysaccharide. Kuzyk *et al.* (1996) presumptively identified one of two carbohydrate antigens of *P. salmonis* as a lipopolysaccharide. Vadovič *et al.* (2007) analysed the structure and composition of lipid A of the *P. salmonis* LPS and determined that it resembled the classical lipid A found in the *Enterobacteriaceae* family.

Host response

The humoral response of coho salmon and rainbow trout infected experimentally with *P. salmonis* was investigated by Kuzyk *et al.* (1996). The infected fish did not produce a strong antibody response and it was most likely directed against a lipooligosaccharide component of the lipopolysaccharide. Arkush *et al.* (2006) showed that while serum anti-*P. salmonis* antibodies were detected in 81.4% of white sea bass surviving experimental exposures to the bacterium, the strength of the response was variable, with an average enzyme-linked immunosorbent assay (ELISA) optical density of 32.8% of the positive control (range 1.7–131.3%) at 123 days post-exposure. Such individual variable responses are not unusual in fish and have been reported for other bacterial and viral fish pathogens. Absence of a significant antibody-mediated response is typical of infections caused by intracellular pathogens and the role of cell-mediated immunity against *P. salmonis* requires further investigation. Recently, microarray-based experiments have been used to identify differentially expressed genes in Atlantic salmon macrophages and anterior kidney cells in response to infection, with promising results (Rise *et al.*, 2004). Nineteen transcripts were selected for their potential utility as biomarkers for research into the molecular pathogenesis of *P. salmonis* infection and in the evaluation of anti-piscirickettsial vaccines and therapeutics. These molecular biomarkers

may also help to develop ideas for treatment approaches. For example, changes were detected in the redox status of infected macrophages, which may enable these cells to tolerate *P. salmonis* infection. It might be possible, then, to reduce haematopoietic tissue damage by antioxidant treatment.

Control, treatment and epizootiology

Although members of the rickettsiae are known to affect mammals (Weiss and Moulder, 1984), *P. salmonis* presents no known health risk to humans. Like many other pathogens of poikilotherms, the inability of the rickettsiae to survive at temperatures of 25°C and above precludes replication at human body temperatures. *P. salmonis* has now been confirmed in two marine fish species and other hosts for this pathogen (or other rickettsia) are likely to be added, as reports of RLOs and PLOs are increasing. Thus, methods for the prevention and treatment of piscirickettsiosis are critical. Simple husbandry practices may facilitate avoidance, including the fallowing of farms in a given region, reduced rearing densities for fish and holding only single year classes at a given site to reduce horizontal transmission. Though vertical transmission has been shown to occur only experimentally (where broodstock were injected with the bacterium; see Larenas *et al.*, 1996, 2003), diagnostic screening of adult broodstock may further reduce disease risk.

Under *in vitro* conditions, *P. salmonis* is sensitive to a broad range of antibiotics, including gentamicin, streptomycin, erythromycin, tetracycline and chloramphenicol (Fryer *et al.*, 1990; Cvitanich *et al.*, 1991). Some resistance to penicillin has been reported. The use of antimicrobial drugs in the treatment of piscirickettsiosis has been reported (Branson and Nieto Díaz-Munoz, 1991; Cvitanich *et al.*, 1991; Smith *et al.*, 1996b), but with inconsistent results (Smith *et al.*, 1996b, 1997). Feeds medicated with oxolinic acid and oxytetracycline seem to be the most effective, but infected fish may be slow to respond and may require repeated applications (Palmer, 1997). The intracellular location of the pathogen may make it possible for substantial numbers of these organisms to be sequestered away from contact with lethal concentrations of antibiotics, and hence maintain the infection.

Vaccines

Several antigens of *P. salmonis* have been detected using rabbit polyclonal antisera and could serve as potential candidates for vaccine development (Kuzyk *et al.*, 1996; Barnes *et al.*, 1998). When convalescent coho salmon and rainbow trout sera were tested against some of the same purified antigens, however, only weak reactions with purified *P. salmonis* antigens were detected by immunoblot, suggesting that the fish humoral response to this pathogen was weak (Kuzyk *et al.*, 1996). Because *P. salmonis* is an intracellular pathogen, stimulation of cell-mediated immunity, including enhanced phagocytosis and intracellular killing, may be critical for success in vaccine development (Barnes *et al.*, 1998).

Initial development of vaccines based on *P. salmonis* bacterins yielded mixed results (Smith *et al.*, 1997; Kuzyk *et al.*, 2001a). In coho salmon, a formalin-inactivated bacterin elicited a dose-dependent response, which in some instances exacerbated the disease, suggesting that the bacterin contained immunosuppressive or other disease-exacerbating factors (Kuzyk *et al.*, 2001a). Birkbeck *et al.* (2004b) showed that vaccines prepared from either heat- or formalin-inactivated *P. salmonis* gave significant protection from *P. salmonis* challenge, with 70.7 and 49.6% relative survival, respectively, compared with 81.8% in the control group. Recently, a highly immunogenic protein, ChaPs, has been obtained from naturally infected coho salmon in southern Chile, and this protein is considered a potential candidate for vaccine development (Marshall *et al.*, 2007). Interestingly, sequence analysis of ChaPs has demonstrated that it is a heat-shock protein, molecules that have already been identified (Wilhelm *et al.*, 2003) and exploited in

recombinant vaccine development (Wilhelm *et al.*, 2006).

Several recombinant vaccines have been tested for piscirickettsiosis control. An outer surface lipoprotein of *P. salmonis*, OspA, produced recombinantly in *E. coli*, conferred good protection in vaccinated coho salmon, with 59% relative per cent survival (RPS) (Kuzyk *et al.*, 2001a). When T-cell epitopes from tetanus toxin and measles virus fusion protein (both of which are immunogenic in mammalian immune systems) were incorporated into an OspA fusion protein, vaccine efficacy increased nearly threefold (83.0% RPS) versus OspA protein alone (30.2% RPS) in a second trial. Another recombinant vaccine, V1, formulated with heat-shock protein (Hsp) 60 and Hsp70 plus the flagellar protein of *P. salmonis*, showed a 95% RPS in Atlantic salmon, while vaccination with formulations containing other *P. salmonis*-derived recombinant proteins, V2 and V3, resulted in 84.4 and 10.4%, respectively (Wilhelm *et al.*, 2006). Reactive antibodies were detected in the sera of fish tested for at least 8 months postvaccination. Efficacy of V1 was later validated in coho salmon (94.5% RPS) by a company that licensed the technology. Earlier attempts using expression library technology were much less successful, perhaps because the proteins used in those trials were not selected based on their association with virulence factors or protective antigens (Miquel *et al.*, 2003). The use of more than one recombinant antigen may potentiate the immune response. Inclusion of Hsp60, for example, may provide a natural adjuvant effect, enhancing a cellular immune response (Wilhelm *et al.*, 2006).

Conclusions and recommendations for future studies

In recent years, significant advances have been made in the development of recombinant vaccines and immunostimulatory compounds for the control of piscirickettsiosis in salmonid fish. Presumably, field trials of these control measures will be conducted with commercialization and subsequent broad-scale application of these treatments to follow. However, key elements of the biology of the agent such as the possibility of a carrier state in previously exposed fish, transmission among and between anadromous salmonids and marine fish species, and environmental or host factors that may precipitate disease outbreaks have yet to be explored fully. Broader geographic and host comparisons of *P. salmonis* and PLO agents via molecular analysis are needed to establish phylogenetic relationships more clearly. Virulence testing of isolates, perhaps coupled with biochemical analysis such as electrophoretic profiling, may help to evaluate pathogenicity. A centrally located culture collection of rickettsiae isolated from fish is needed to facilitate such molecular, biological and antigenic comparisons. With the intriguing finding that *P. salmonis* can now be isolated directly on certain agar or broth media, early detection of the bacterium should facilitate rapid diagnosis and mitigate losses caused by this pathogen.

Francisella

The disease agent

Aetiological agent

Francisella sp. are non-motile, Gram-negative, facultatively intracellular bacteria that form coccoid to short rods and are strict aerobes. Ultrastructure studies reveal extremely irregular, pleomorphic bacteria with a size of $1.45 \times 0.35\,\mu m$ within the cytoplasmic vacuoles of phagocytes (Hsieh *et al.*, 2006).

The family *Francisellaceae* belongs to the gamma subclass of the Proteobacteria and contains the single genus *Francisella*, with two recognized species: *F. tularensis* and *F. philomiragia*. In 2002, Kamaishi *et al.* (2005) demonstrated that the 16S rRNA gene sequence of an RLO isolated from three-lined grunt *Parapristipoma trilineatum* (Fukuda *et al.*, 2002) had high similarity to that of *F. philomiragia*. Comparison of

16S rRNA genes from similar bacteria infecting Atlantic cod (*Gadus morhua*) and tilapia (*Oreochromis* spp.) from Asia and Latin America also showed high similarity to *F. philomiragia* (Nylund *et al.*, 2006; Hsieh *et al.*, 2007; Mikalsen *et al.*, 2007; Ottem *et al.*, 2007), yet the isolates from cod were distinct from established *Francisella* species, as well as the isolates from tilapia and grunt. Two names have been proposed for the isolates from cod: Mikalsen *et al.* (2007) proposed the new subspecies, *F. philomiragia* subsp. *noatunensis* subsp. nov., and Ottem *et al.* (2007) proposed the new species, *F. piscicida* sp. nov. In addition, the name *F. victoria* was proposed for a pathogen isolated from tilapia (Kay *et al.*, 2006); however, the source of this isolate and its 16S rRNA gene sequence were not published.

The organisms are fastidious and most, but not all, isolates have a high requirement for cysteine (Hsieh *et al.*, 2006; Ottem *et al.*, 2006). When cultured on cysteine heart agar with 5% sheep blood (CHAB), growth occurred between 10 and 30°C and was optimal at 22°C (Olsen *et al.*, 2006; Mikalsen *et al.*, 2007); in comparison, *F. philomiragia* isolates were able to grow at 35°C.

Transmission

Horizonal transmission was demonstrated in cod (Nylund *et al.*, 2006) by cohabitation of infected and uninfected fish. On the basis of histological and ISH examination of infected tilapia, the digestive tract may be the primary route of entry and transmission in that species is suggested to be by the oral–faecal route (Hsieh *et al.*, 2007).

Nylund *et al.* (2006) speculate that the bacteria may be introduced into cod farms through fry reared from wild brood, or that natural reservoirs may exist in the area of the farms in the form of amoeba, wild fish or other marine species. Detection of *Francisella* sp. in tissues of cod, saithe (*Pollachius virens*), blue mussel (*Mytilus edulis*) and edible crab (*Cancer pagurus*) from the vicinity of affected cod farms (Ottem *et al.*, 2006) suggests that there is high potential for transmission between farms. The ability of these organisms to survive for prolonged periods in the aquatic environment also increases the risk of transmission; however, there have as yet been no studies that have examined vertical transmission or other vectors.

Geographical distribution and host range

Infections caused by organisms identified as *Francisella* sp. have been reported from three-lined grunts in Japan (Fukuda *et al.*, 2002; Kamaishi *et al.*, 2005), ornamental cichlids in Taiwan (Hsieh *et al.*, 2007), tilapia in Taiwan (Hsieh *et al.*, 2006) and Latin America (Mauel *et al.*, 2007), Atlantic cod in Norway (Nylund *et al.*, 2006; Olsen *et al.*, 2006) and hybrid striped bass (*Morone chrysops*×*M. saxatilis*) in California (Ostland *et al.*, 2006). The recent diagnosis of *Francisella* sp. infection in Atlantic salmon reared in fresh water in Chile appears to be the same agent described over a decade earlier by Cvitanich *et al.* (1995) (Birkbeck *et al.*, 2007). This suggests a likely global distribution and broad host species range.

Infections caused by similar fastidious intracellular bacteria have been reported from other fish; however, because definitive identification has not been made, these are considered in the section on RLOs and FLB that follows. The relationships between these organisms will become clearer as additional gene sequences become available for comparison and as the physiochemical characteristics of the different isolates are determined.

Economic importance of the disease

Infections by *Francisella* sp. have been reported only recently; however, severe granulomatous disease has been reported from several economically important species. Epizootics were reported in Nile tilapia from three facilities in Latin America (Mauel *et al.*, 2007) and similar outbreaks by *Francisella* sp. or FLB have occurred in farmed tilapia in Taiwan, Jamaica, Indonesia, Florida and southern California (Hsieh *et al.*, 2006; Mauel *et al.*, 2007). Ornamental

cichlids appear particularly susceptible to the infection and Hsieh *et al.* (2007) suggest that this bacterium is a major pathogen for farmed tilapia and ornamental cichlids.

For cod, a newly cultured species in Norway, this bacterium was associated with the first major disease and occurred at several production sites (Nylund *et al.*, 2006; Mikalsen *et al.*, 2007). Over a 5-month period, mortalities reached about 40%, with larger fish being most affected (Olsen *et al.*, 2006). In Chile, an outbreak of granulomatous disease in Atlantic salmon parr held in freshwater net pens was attributed to *Francisella* sp. (Birkbeck *et al.*, 2007) and linked to earlier observations of an intracellular bacterium morphologically distinct from *P. salmonis* (Cvitanich *et al.*, 1995). The similarity of the isolates from cod and Atlantic salmon raise questions about the virulence of this pathogen for these species, its presence in both fresh water and salt water and the role of wild stocks of these fish as reservoirs of infection.

Although *F. philomiragia* has been reported as a cause of opportunistic infection in humans, there have been no reported human cases of infection as a result of outbreaks of FLB in fish. The lower temperature tolerance of the isolates infecting fish and their more fastidious metabolic requirements suggest that these are unlikely to cause disease in warm-blooded animals (Mauel *et al.*, 2007; Mikalsen *et al.*, 2007).

Diagnostic methods

Gross morphological features of the infection are not unique in appearance and are easy to confuse with other infections such as mycobacteriosis, nocardiosis and fungal infections (Hsieh *et al.*, 2007), and presumptive diagnosis of *Francisella* infection is typically established by histopathological examination. Bacteria are found in the cytoplasm of infected macrophages rather than in a cytoplasmic vacuole, which may help in differentiating from *P. salmonis* infection.

The agent has been cultured in CHSE-214, salmon head-kidney (SKH-1) and Atlantic salmon kidney (ASK) (Hsieh *et al.*, 2006; Nylund *et al.*, 2006), with the bacteria causing a CPE of rounding and clustering of cells. The bacterium, although fastidious, has been grown on CHAB. Success of culture on other agar media, including blood agar plates containing 0.1% cysteine and 1% D-glucose (BCG agar), trypton soy agar (TSA), brain–heart infusion agar (BHIA), chocolate agar and Thayer–Martin agar, has been variable. Two liquid media have also been described for culture, Bacto™ Eugon broth containing $FeCl_3$ and B1817 medium consisting of Marine Broth 2216 (Difco) supplemented with fetal calf serum, yeastolate ultrafiltrate, cysteine and D-glucose (Kamaishi *et al.*, 2005; Ottem *et al.*, 2006). When cultured on CHAB plates, bacterial colonies from isolates of Atlantic salmon and cod are low convex, whitish, slightly translucent and mucoid in appearance (Mikalsen *et al.*, 2007). Colonies of isolates from tilapia cultured on CHAB were of similar morphology, except that they were more mucoid and pale yellow in coloration (Birkbeck *et al.*, 2007), whereas when cultured on Thayer–Martin agar they appeared smooth with greyish pigment (Hsieh *et al.*, 2006). Comparative studies have not been conducted to evaluate the ability of enriched media to support growth of isolates from different species, nor has the sensitivity and specificity of the enriched blood agar media for primary isolation from fish tissues versus cell culture methods been assessed.

Phenotypic differences were also detected in alkaline phosphatase (AP) activity, with isolates from tilapia and three-lined grunt being AP-positive and isolates from Atlantic cod and Chilean Atlantic salmon AP-negative (Birkbeck *et al.*, 2007). However, characterization of the isolates from tilapia and Atlantic cod showed this bacterium was distinct from *F. philomiragia* by testing cytochrome oxidase negative (Hsieh *et al.*, 2006; Mikalsen *et al.*, 2007; Ottem *et al.*, 2007).

ISH and PCR assays were compared for detection of the organism in ornamental cichlids, with ISH providing greater sensitivity in cases where smaller numbers of bacteria were present (Hsieh *et al.*, 2007). Although PCR has been used extensively for amplification of the 16S rRNA for sequence

analysis of isolates from the different species, primers have not been tested for diagnostic purposes.

Genome structure and transcription

Comparisons of 16S rRNA gene sequences are just beginning to resolve relationships among these isolates. An isolate from Atlantic salmon in Chile showed 99.8% sequence identity with the cod isolate (Birkbeck *et al.*, 2007). Isolates from tilapia from Latin America appear similar to other isolates from three-lined grunt and tilapia (99.9% sequence identity with 16S rDNA sequence of isolates from tilapia in Taiwan) and to the isolate from cod (99.2% similar) (Mauel *et al.*, 2007). The relationship between these organisms will become clearer as additional gene sequences become available for comparison and as the physiochemical characteristics of the different isolates are elucidated. Other genes that may provide additional information include the 16S–23S rRNA intragenic spacer and the partial outer membrane protein (FpoA) gene.

Pathogenesis and immunity

Disease

Fukuda *et al.* (2002) described infected tilapia having haemorrhage on the body surface, gill and ventral region. Other descriptions of tilapia infected by *Francisella* sp. include dark or pale coloration, abnormal swimming behaviour, petechiae or skin ulcerations and exophthalmia. In Atlantic cod and hybrid striped bass, loss of appetite, reduced swimming performance and dark pigmentation were common disease signs. Raised haemorrhagic nodules were sometimes visible on the skin (Olsen *et al.*, 2006; Ostland *et al.*, 2006) and white granulomas could be found on the skin, gills and in the mouth cavity (Nylund *et al.*, 2006). Internal signs of disease seem to be similar across the species infected. Enlargement of the spleen, kidney and heart is common. Whitish nodules, or granulomas, are visible on most visceral organs, particularly the kidney and spleen. Opacity of one or both eyes was observed in some fish (Olsen *et al.*, 2006).

Pathogenesis

Histopathologic assessment of infection in experimentally infected hybrid striped bass indicates that the bacterium quickly enters the intracellular environment (Ostland *et al.*, 2006). The target cells seem to be phagocytes and cells having phagocytic functions (Nylund *et al.*, 2006; Hsieh *et al.*, 2007). Histopathological descriptions of the disease from tilapia, three-lined grunt, striped bass and cod are similar. In the early stages of disease in cod, the bacteria were detected from phagocytes in the kidney and spleen, as well as in endothelial cells lining the heart chamber and leucocytes attached to blood vessel walls in the liver, pseudobranch and gills. As the disease progressed, granulomas consisted mainly of host cells with few bacteria present. The predominating cells were hypertrophied, foamy macrophages. In terminal stages, the core of the granuloma becomes necrotic, the cells in the centre die and are replaced by a transparent liquid and bacteria can no longer be detected (Nylund *et al.*, 2006; Olsen *et al.*, 2006).

In hybrid striped bass, additional histopathological changes were observed in the heart and gills. In the heart, there was extensive endothelial hypertrophy and hyperplasia, but bacteria were not commonly observed. In the gills, a mononuclear inflammatory infiltrate was seen in the interstitium of the gill filaments, with intracellular bacteria within some of these cells (Ostland *et al.*, 2006).

Virulence factors

Little is known of the virulence mechanisms of the *Francisella* species. The inability to detect the bacterium within a distinct cytoplasmic vacuole may result from the ability of the pathogen to alter the phagosomal maturation process and enter the cytoplasm of the macrophage, as has been described

during infections by *F. tularensis* (Clemens *et al*., 2004; Ostland *et al*., 2006).

The LPS of *F. victoria* was described as containing two variants, neither of which had reciprocal cross-reactivity with the LPS of *F. tularensis* or *F. philomiragia*. However, structural elements associated with *F. victoria* LPS are characteristic of the genus and may play a role in how these species evade or suppress the host immune response effectively (Kay *et al*., 2006).

Host response

The host responds to infection by formation of granulomas (Fig. 8.7). Other components of the host immune response have not yet been examined.

Control, treatment and epizootiology

Transmission control

The widespread geographic distribution, broad host range and fish–fish transmission of *Francisella* sp. suggest environmental reservoirs that will make it difficult to control or predict outbreaks by these pathogens.

Chemotherapy

Mortality associated with FLB in striped bass has been controlled with oxytetracycline administered at 3.8 g/0.45 kg of food when fed at 3% body weight/day for 10 days (Ostland *et al*., 2006). However, it could be expected that treatment of infections by *Francisella* sp. will encounter difficulties similar to those in treating *P. salmonis*. The difficulties in treating bacteria that survive in the intracellular environment make vaccines a logical alternative.

Conclusions and recommendations for future studies

The recognition that these organisms are distinct from *P. salmonis* and other PLOs is only recent and it is likely that the increasing ability of molecular detection methods will provide a means to resolve the aetiology of past disease outbreaks. The molecular

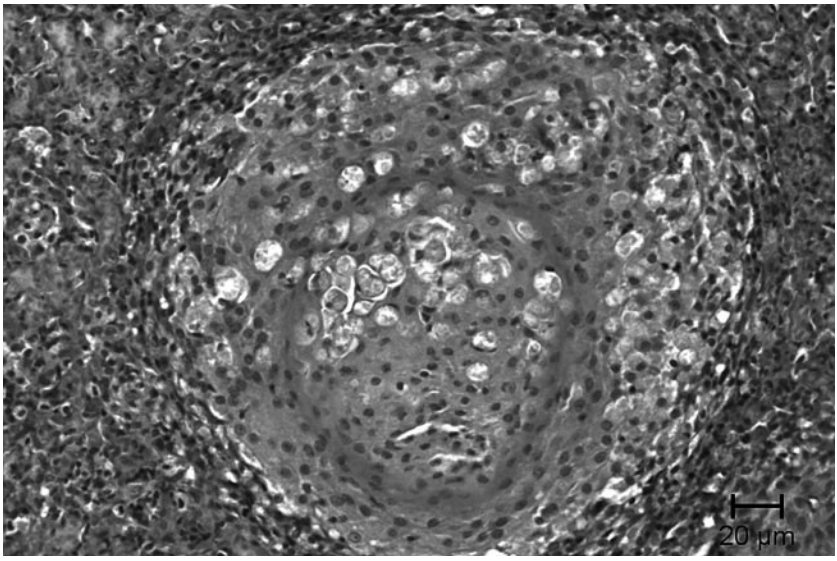


Fig. 8.7. Granulomatous inflammation in spleen of tilapia *Oreochromis* sp. with *Francisella*-like bacteria. Photograph courtesy of Andrew Goodwin, University of Arkansas, Pine Bluff, USA.

studies should continue, but should be combined with comparative phenotypic studies to resolve relationships among the different isolates. Additional comparative work on diagnostic culture methods is also needed to define optimal media and conditions for this group and, because these agents can be cultured, it will also be feasible to test therapeutic methods. Laboratory trials should be conducted to determine host specificity for isolates from different species. All of these studies would be facilitated by having a centralized repository for these agents as they are isolated.

Because of the apparent broad distribution of these organisms and the potential for an environmental reservoir, it will be necessary to determine the mode(s) of transmission in order to develop risk assessments for developing aquaculture species.

Other Rickettsia-like Organisms and *Francisella*-like Bacteria

The earliest report of an RLO in fish was by Mohamed (1939), who observed the organism in cells of diseased *Tetrodon fahaka* taken from the Nile River, Egypt. Diagnosis was made on the basis of staining and intracellular location; however, the organism was not cultured. The second observation of an RLO was from cultured fish cells inoculated with tissue homogenates from rainbow trout in Europe (Ozel and Schwanz-Pfitzner, 1975). These fish were also infected with viral haemorrhagic septicaemia virus, so the relationship of the RLO to the mortality that occurred in the trout population was unknown. Studies were not conducted to verify the identity of the microorganism or to determine its pathogenicity. In 1986, Davies observed an RLO in the tissues of dragonets (*Callionymus lyra*) collected in waters off the coast of Wales (Davies, 1986). An RLO was observed in moribund specimens of the blue-eyed plecostomus (*Panaque suttoni*) imported into the USA from Colombia, South America (Khoo *et al.*, 1995). In France, an RLO was observed in the brain of juvenile sea bass (*D. labrax*) exhibiting abnormal swimming behaviour (Comps *et al.*, 1996). The identity of many of these organisms will remain unresolved, but for others there are sufficient serological or morphological data to support their designation as an RLO. For example, immunohistochemical comparisons of an RLO from sea bass (*D. labrax* L.) in Greece confirmed its similarity to *P. salmonis* (Athanassopoulou *et al.*, 2004). Antigenic similarities of other European sea bass RLOs to *P. salmonis* have been demonstrated (Steiropoulos *et al.*, 2002; Timur *et al.*, 2005). In China during 2001–2002, an emerging disease seen in snakehead (*Ophiocephalus argus*) was reported and electron microscopic observations were used to confirm that the causative agent was an RLO (Guo *et al.*, 2004).

In contrast to isolates for which there was evidence for antigenic similarities or morphological characteristics that were in common with an RLO, an unidentified agent (UA2) similar to *P. salmonis*, but smaller in size, was isolated in fish cell cultures from Atlantic salmon reared in fresh water in Chile (Cvitanich *et al.*, 1995). The agent was not recognized by anti-*P. salmonis* antibodies in a direct fluorescent antibody test and the work by Birkbeck *et al.* (2007) indicated that this agent was likely to be a *Francisella* sp. Similarly, links to the genus *Francisella* have been made to various outbreaks which have occurred in tilapia. In the early 1990s, a disease outbreak among tilapia in Taiwan was linked directly to an RLO (Chen *et al.*, 1994; Chern and Chao, 1994). The disease eventually spread to approximately 37 rearing facilities in both fresh water and salt water; six species of tilapia were affected and mortality reached 95% at certain sites. A PLO in tilapia caused severe mortalities in cultured and wild tilapia in Hawaii, prompting control measures designed to prevent transfer of infected tilapia from Oahu to other Hawaiian islands (Mauel and Miller, 2002; Mauel *et al.*, 2003). A similar bacterium was described from epizootics in tilapia in Florida, California and South Carolina (Mauel *et al.*, 2005). Behavioural signs and pathologies exhibited during each of these outbreaks were similar to those in salmon infected with *P. salmonis*. However,

the bacterium was smaller than *P. salmonis*, did not grow using standard culture techniques and *P. salmonis*-specific primers did not amplify bacterial DNA. Subsequently, genetic analysis of similar bacteria in tilapia in Taiwan and Latin America (Hsieh *et al.*, 2007; Mauel *et al.*, 2007) has revealed a closer affinity with the *Francisella*.

The increasing availability of molecular diagnostic methods is facilitating the identification of disease agents that are difficult to isolate in culture. These methods might also offer some insight on historical observations of these organisms, many of which were classified provisionally as RLOs.

Epitheliocystis

The disease agent

The causative agent(s) of epitheliocystis (EP) is(are) morphologically diverse and may represent a group of related organisms that produce similar pathology in both marine and freshwater fish. These organisms are intracellular, Gram-negative bacteria. On the basis of their ultrastructure (Paperna *et al.*, 1981; Groff *et al.*, 1996; Szakolczai *et al.*, 1999) and 16R rRNA sequence (Draghi *et al.*, 2004; Meijer *et al.*, 2006; Nowak and LaPatra, 2006; Karlsen *et al.*, 2008), they fit in the order *Chlamydiales* (Moulder, 1984).

Molecular evidence indicates a high degree of diversity among the fish pathogenic *Chlamydiae* that may be related to host specificity (Meijer *et al.*, 2006). *Chlamydiales* from salmonids have been classified as *Neochlamydia* sp. from Arctic charr (*S. alpinus*) (Draghi *et al.*, 2007) and '*Candidatus* Piscichlamydia salmonis' (Draghi *et al.*, 2004) and '*Candidatus* Clavochlamydia salmonicola' (Karlsen *et al.*, 2008) from Atlantic salmon. Identification of three novel *Chlamydiales* from leafy seadragon (*Phycodurus eques*), silver perch (*Bidyanus bidyanus*) and barramundi (*Lates calcarifer*) (Meijer *et al.*, 2006) indicates that inclusion of additional isolates from non-salmonid species will be necessary for clarifying the taxonomy of this group. Similarly, the inability to demonstrate the presence of a genus-specific, lipopolysaccharide antigen in all cases of EP observed in fish supports a wide taxonomic variation in this group (Nowak and LaPatra, 2006).

Due to the inability to isolate these organisms, characterization is based on observations of their ultrastructure. The bacteria are pleomorphic and range from about 0.2 to 1.2 µm in diameter. Two chlamydial developmental cycles have been described from fish. One is the typical cycle that alternates between a non-replicating infectious elementary body and a replicating, non-infectious reticulate body (Avaykan and Popov, 1984; Corsaro and Greub, 2006). In this cycle, infection is initiated by attachment of the elementary body to the host cell. Once the bodies enter the cell, they germinate and convert to the reticulate form, which is internalized with endocytic vesicles. After division, the organism reverts to its elementary body form and is released from the cell by exocytosis. In the second cycle, intracellular vegetative stages are identified as primary and intermediate long cells, with the infective form being small cells (Crespo *et al.*, 1999). Stages from both may be found in the same fish species, suggesting that the developmental stage is influenced by fish condition, environmental factors or cell type infected rather than being characteristic of the bacterium itself (Paperna *et al.*, 1981; Paperna and Alves de Matos, 1984; Crespo *et al.*, 1999).

Transmission

Natural transmission of these organisms is not understood, but horizontal transmission apparently occurs within some host species. Hoffman *et al.* (1969) described experiments in the bluegill and Wolf (1988) listed experiments conducted by D.S. Wyand in goldfish (*Carassius auratus*) that demonstrated direct transmission. In both studies, EP developed in the experimental animals 3–4 weeks after the addition of infected gill tissue to aquaria where uninfected fish were

held. Paperna (1977) suggested that transmission from infected fish might occur in culture facilities through contaminated nets and other equipment.

The genetic similarity of the chlamydia associated with fish and the 'novel' chlamydia associated with free-living amoeba suggests a possible reservoir of infection (Fritsche *et al.*, 2000; Corsaro and Greub, 2006; Draghi *et al.*, 2007). However, there is as yet no experimental evidence for this and in surveys conducted during outbreaks of amoebic gill disease in Atlantic salmon in Tasmania, EP is rare (Nowak and Clark, 1999).

Geographical distribution and host range

EP was first described in the bluegill sunfish (*Lepomis macrochirus*) by Hoffman *et al.* (1969), who identified the aetiological agent as a bedsonia (now termed chlamydia). Molnar and Boros (1981) verified the chlamydia-like nature of the causative agent and determined that EP was identical to the mucophilosis disease of common carp described by Plehn (1920) and believed to be caused by a unicellular alga or fungus. These descriptions were followed by reports of the disease in freshwater, marine and anadromous fish from both warmwater and coldwater environments. To date, the documented host range includes more than 50 species and over 23 families, including members of the Acipenseridae (Groff *et al.*, 1996), Carangidae (Crespo *et al.*, 1990; Venizelos and Benetti, 1996), Centrarchidae (Hoffman *et al.*, 1969), Cichlidae (Paperna and Sabnai, 1980), Cyprinidae (Plehn, 1920), Gadidae (Lewis *et al.*, 1992), Ictaluridae (Zimmer *et al.*, 1984; Desser *et al.*, 1988), Moronidae (Wolke *et al.*, 1970; Paperna and Zwerner, 1976; Paperna and Baudin Laurencin, 1979), Mugilidae (Paperna and Sabnai, 1980; Paperna *et al.*, 1981), Mullidae (Paperna and Sabnai, 1980), Oplegnathidae (Egusa *et al.*, 1987), Paralichthyidae (Lewis *et al.*, 1992) and Percidae (Anderson and Prior, 1992), among others. It is likely that additional hosts have gone unreported because of the self-limiting nature of the disease. Because a laboratory challenge model has not been developed for this disease, host susceptibility has been inferred from surveys and is likely underestimated because of species targeted in these studies and limited sample size.

Economic importance of the disease

Often, EP is regarded as relatively benign, but severe mortality has been associated with the infection in cultured fish, typically during early life stages (Nowak and LaPatra, 2006).

Diagnostic methods

Preliminary diagnosis of EP is made by observation of the white to yellow cysts on the gills or skin of affected fish (Wolf, 1988). The thick capsule and granular contents, which are characteristic of EP cysts, are seen easily in wet mounts (Fig. 8.8), but histology is recommended for definitive diagnosis (Nowak and LaPatra, 2006). In histological sections, the granular basophilic inclusion contains large numbers of coccoid or coccobacillary cells (Fig. 8.9). When the cell nucleus is visible, it is on the periphery of the cell, a characteristic that distinguishes the infection from lymphocystis virus infection (Noga, 2000). The gill is the organ affected most frequently, but it has been suggested that the pseudobranch also be examined for cysts (Crespo *et al.*, 1990).

Increasingly, molecular methods are being used to detect EP infection, and both PCR and ISH using *Chlamydia*-specific primers have been used in several studies (Draghi *et al.*, 2004, 2007; Meijer *et al.*, 2006; Karlsen *et al.*, 2008). Several pan-chlamydia primer sets have been designed from the 16S rRNA gene (Corsaro and Greub, 2006; Meijer *et al.*, 2006); however, because of the genetic diversity within the group, there are no universally accepted primers for diagnostic purposes at this time.

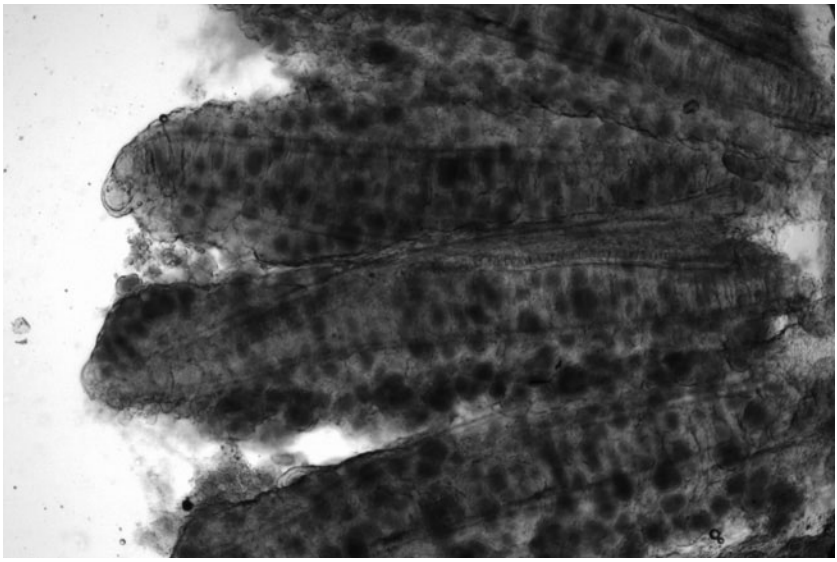


Fig. 8.8. Wet mount of gill filaments from largemouth bass (*Micropterus salmoides*) with epitheliocystis. Photograph courtesy of Andrew Goodwin, University of Arkansas, Pine Bluff, USA.

Pathogenesis and immunity

Disease

EP occurs as a benign or proliferative disease, characterized by cysts in the branchial and (rarely) skin epithelia of the host. Clinical signs resulting from infection in the gills may include lethargy, flared opercula and rapid respiration. Cysts may appear as transparent white to yellow nodules, up to approximately 1 mm in diameter on the gill filaments (Fig. 8.8). Generally, the host response is limited, and there is little or no mortality associated with infection. However, when host responses are proliferative, hyperplasia of the branchial epithelium can cause respiratory insufficiency, and mortality associated with this condition has been as high as 100% in cultured juvenile fish (Nowak and LaPatra, 2006; Draghi *et al.*, 2007).

Characteristic cysts are hypertrophic host cells filled with the causative bacterium (Fig. 8.9). The enlarged host cells range from 10 to 400 µm in diameter and frequently are surrounded by squamous or cuboidal epithelial cells (Fig. 8.9). The nature and origin of the target host cell are unclear; cysts may originate from a single cell (Zachary and Paperna, 1977) or be a coalescence of several (Wolke *et al.*, 1970). The infected cells are typically epithelial; however, infection has been described variously in mucus, epithelial lining and chloride cells of an unidentified species of carp (Paperna and Alves de Matos, 1984); in pillar cells of red sea bream (*Pagrus major*) and tiger puffer (*Takifugu rubripes*) (Miyazaki *et al.*, 1986); and in cells described as possible transformed macrophages in brown bullhead (*Ictalurus nebulosus*) (Desser *et al.*, 1988). Molnar and Boros (1981) described the development of EP cysts in the gills of the common carp (*Cyprinus carpio*). In the early stages of the disease, infected cells are 10–15 µm in diameter and contain a central inclusion body, foamy cytoplasm and eccentric nucleus. As infection progresses, infected cells increase in size to 70–80 µm in diameter and the granular inclusion fills the entire host cell, compressing or replacing both the nucleus and the cytoplasm.

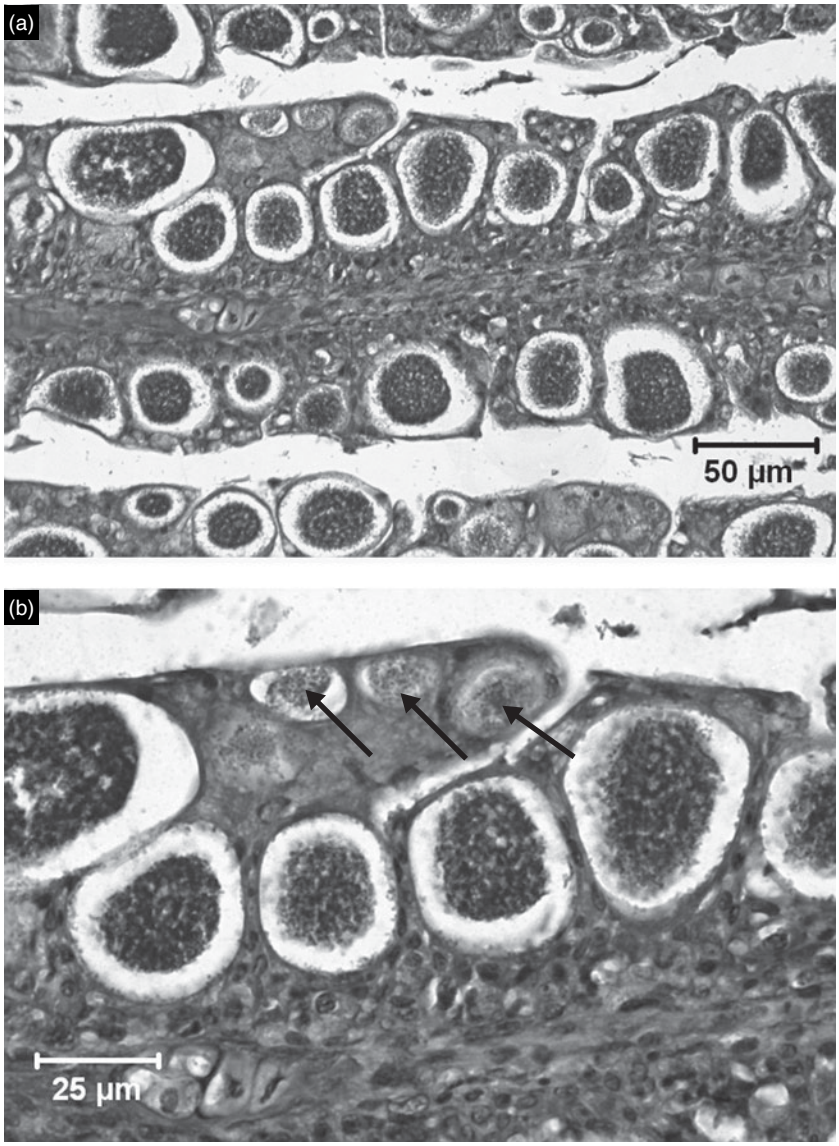


Fig. 8.9. (a) Histological section of largemouth bass (*Micropterus salmoides*) gill with epitheliocystis. Cysts fill the interlamellar spaces. (b) Higher magnification of gill showing development of the agent in the host cells (arrows). Photographs courtesy of Andrew Goodwin, University of Arkansas, Pine Bluff, USA.

In benign infections, cysts may be surrounded by a layer of squamous or cuboidal epithelial cells. Typically, no host response is apparent, even in the presence of large numbers of cysts. However, in certain cases, the organism induces an extensive host response, with unrestricted proliferation of gill epithelia and extensive mucus production, a condition referred to as hyperinfection (Paperna, 1977; Rourke *et al.*, 1984; Bradley *et al.*, 1988). In proliferative infections, hyperplastic epithelial cells form

concentric layers around the cysts and proliferate throughout the gill filaments. Infiltrating macrophages and eosinophils combine with the hyperplastic tissue to surround and obstruct the capillary network of the gill filament. These circumstances impair both gas transfer and osmoregulatory processes, which may result in the death of infected fish. Induction of a cellular response, however, does not appear to be influenced by the size and location of the cyst in the gill filament (Nowak and Clark, 1999).

The proliferative form of the disease has been described from a variety of freshwater and marine species, including lake charr (*S. namaycush*) (Bradley *et al.*, 1988), Arctic charr (Draghi *et al.*, 2007), largemouth bass (*Micropterus salmoides*) (Goodwin *et al.*, 2005), pacu (*Piaractus mesopotamicus*) (Szakolczai *et al.*, 1999), red sea bream (Miyazaki *et al.*, 1986), greater amberjack (*Seriola dumerili*) (Crespo *et al.*, 1990), gilthead sea bream (*Sparus aurata*) (Paperna, 1977) and Atlantic salmon (Nylund *et al.*, 1998), indicating that a proliferative response to infection is not related to host species or geographical location (Nowak and LaPatra, 2006). The causes for variation in the severity of EP infections are not clear, but proliferative infections have been detected in cultured fish only and mortality associated with this condition has been as high as 100% (Paperna, 1977; Miyazaki *et al.*, 1986; Bradley *et al.*, 1988; Crespo *et al.*, 1990; Nowak and LaPatra, 2006; Draghi *et al.*, 2007). However, high mortality has also been reported in the absence of a proliferative host response in several species including white sturgeon (*Acipenser transmontanus*) (Groff *et al.*, 1996), yellowtail amberjack (*S. lalandi*) (Venizelos and Benetti, 1996; Nowak and LaPatra, 2006), flathead (*Platycephalus* sp.) (Miyaki *et al.*, 1998), sharpsnout sea bream (*Diplodus puntazzo*) (Katharios *et al.*, 2008) and Australian bass (*Macquaria novemaculeata*) (Nowak and LaPatra, 2006). While observations of proliferative infections in cultured fish only may be an artefact resulting from the increased surveillance of captive species, it suggests that culture conditions may exacerbate the infection.

Benign infections are found more frequently in free-ranging fish (Grau and Crespo, 1991). Progression from the chronic to the progressive form may occur when host defence mechanisms are compromised by genetics or environmental factors (Paperna and Sabnai, 1980). Nowak and LaPatra (2006) suggest that increased concentrations of nutrients, higher fish densities and/or stress associated with aquaculture may increase the prevalence or severity of EP. An association was made between season and prevalence of EP in Atlantic salmon in Tasmania, with infection higher during summer months when water temperatures were higher (Nowak and Clark, 1999), and this was supported by observation of high mortality in sharpsnout sea bream in Crete when water temperatures were high (Katharios *et al.*, 2008); however, studies on other species indicate progression of EP from a chronic to proliferative form with low temperature (Crespo *et al.*, 1990; Turnbull, 1993). The effects of age have also been contradictory, with one study suggesting a positive correlation between EP prevalence in wild winter flounder (*Pleuronectes americanus*) (MacLean, 1993) and another showing no relation to age in cultured Atlantic salmon (Nowak and Clark, 1999). Because the causative organism has not been isolated, the possibility that these observations can be explained by differences in virulence of the pathogen cannot be excluded.

Co-infections with a variety of infectious agents and parasites have also been reported, but their role in EP is not clear. For example, co-infections have been reported with Atlantic salmon paramyxovirus in Atlantic salmon (Kvellestad *et al.*, 2003, 2005), *Trichodina* sp. in Atlantic salmon (Nylund *et al.*, 1998) and red sea bream (Syasina *et al.*, 2004), and lymphocystis in barramundi (Meijer *et al.*, 2006). Nowak and LaPatra (2006) cite several examples of co-infection with *Gyrodactylus* sp. and other gill monogeneans and suggest that this association may represent a link between the two infections or that the same risk factors are involved.

Control, treatment and epizootiology

Sterilization of rearing water using ultraviolet light has been reported to control outbreaks of EP in amberjack (*S. dumerili*) and coral trout (*Plectropomus leopardus*) (Miyaki *et al.*, 1998).

Chemotherapy

Oxytetracycline (25 mg/l active ingredient) administered twice daily for 3 days treated EP in farmed largemouth bass successfully (Goodwin *et al.*, 2005). Historically, Paperna (1977) reported treating hatchery-reared juvenile *S. aurata*, suspected of having EP, by feeding 1% chloramphenicol mixed in ground chicken livers. Although no information on untreated fish was included, it was implied that the infection was controlled by the antibiotic therapy.

Vaccines

Development of vaccines against EP is unlikely at this time. The infection is frequently benign, the aetiological agents appear diverse and none have been isolated or grown in culture. Mortality associated with EP occurs principally in hatcheries, where prevention and control of the disease can be accomplished best through careful attention to proper fish husbandry.

Conclusions and recommendations for future studies

Molecular studies indicate that this is a highly diverse group with host-species specificity. If true, this implies that transmission between species is limited and this has implications for management of infections. However, until the agent can be cultured, cross-infection trials are not feasible and thus molecular data will continue to be used to infer these relationships. Future studies should be directed towards *in vitro* propagation of the aetiological agents, determination of taxonomic placement and clarification of the relationship among morphologically diverse groups of these organisms.

The mode(s) of transmission should be clarified, and identification of the factors leading to development of a proliferative rather than a benign infection is a priority. Studies on the antimicrobial sensitivity of the aetiological agent(s) and development and testing of therapeutic procedures for control of the proliferative infection are important. Development of more rapid and precise diagnostic procedures for identification of the disease would also be helpful.

Acknowledgements

This chapter is dedicated to John L. Fryer (4 July 1926 to 31 August 2004), who, for more than 40 years, headed a programme on infectious diseases of Pacific salmon at Oregon State University (USA). Among his many research accomplishments was the characterization of *P. salmonis*. The authors thank R.P. Hedrick of the School of Veterinary Medicine, University of California, for critical review of this chapter and Inter-Research for permission to reprint images. This is BML Contribution Number 2435, Bodega Marine Laboratory, University of California at Davis.

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9 Bacterial Kidney Disease (*Renibacterium salmoninarum*)

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Introduction

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, is a prevalent disease that impacts the sustainable production of salmonid fish for consumption and species conservation efforts. The disease is chronic in nature and mortality most often occurs in 6- to 12-month-old juvenile salmonids and pre-spawning adults (Evelyn, 1993). The bacterium is a small (0.3–0.1 μm×1.0–1.5 μm), non-motile, non-spore-forming, non-acid-fast, Gram-positive diplobacillus (Fryer and Sanders, 1981), and there is only one species described in the genus. Research on *R. salmoninarum* has been impeded, due, in part, to technical difficulties of working with this slow-growing and fastidious microorganism. The genome sequence of strain ATCC 33209 has been determined and has provided some insight into the evolution, genetic structure and metabolism of the pathogen (Wiens *et al.*, 2008).

The kidney disease bacterium can be transmitted horizontally from infected fish sharing a water supply (Mitchum and Sherman, 1981; Bell *et al.*, 1984) and is one of the few examples of a vertically transmitted bacterial pathogen of vertebrates via association with eggs from infected parents (Evelyn *et al.*, 1986b; Pascho *et al.*, 1991b). The spread of BKD has followed the expansion of salmonid culture (Rohovec and Fryer, 1988; Evenden *et al.*, 1993) and most recorded outbreaks of BKD have occurred in fish culture facilities. Losses as high as 80% in stocks of Pacific salmon (*Oncorhynchus* spp.) and 40% in stocks of Atlantic salmon (*Salmo salar*) have been reported (Evenden *et al.*, 1993). Salmonids vary in their susceptibility to BKD: Pacific salmon species are the most susceptible, while Atlantic salmon and rainbow trout (*O. mykiss*) are considered more resistant (Sakai *et al.*, 1991; Evelyn, 1993; Fryer and Lannan, 1993; Starliper *et al.*, 1997). The disease has also been documented in naturally spawning populations that have never been supplemented with hatchery fish (Evelyn *et al.*, 1973; Souter *et al.*, 1987). The chronic nature of the disease has hindered accurate estimates of fish losses, particularly in feral fish populations (Pippy, 1969; Maclean and Yoder, 1970; Mitchum *et al.*, 1979; Banner *et al.*, 1986). BKD is an important cause of infectious disease-related mortality in restoration and conservation programmes for endangered sockeye (*O. nerka*) and Chinook (*O. tshawytscha*) salmon in the USA (Flagg and Mahnken, 1995; Hoffnagle *et al.*, 2003).

As with other infectious diseases of salmonids that are difficult or impossible to

© CAB International 2011. *Fish Diseases and Disorders* Vol. 3: *Viral, Bacterial and Fungal Infections*, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

treat, avoidance is recommended for the control of BKD in cultured salmonid stocks (Evelyn, 1993). Because *R. salmoninarum* can be enzootic in wild salmonid populations (Evelyn *et al.*, 1973; Jónsdóttir *et al.*, 1998; Bruneau *et al.*, 1999), measures to control losses from BKD may be near impossible as hatchery fish may be exposed constantly to waterborne bacteria shed into the water supply by wild fish residing upstream from the hatchery (Mitchum and Sherman, 1981; Hastein and Lindstad, 1991). Broodstock segregation or culling is now used to select egg lots to retain as a source of juvenile fish for hatchery rearing (Pascho *et al.*, 1991b; Warren, 1991; Gudmundsdóttir *et al.*, 2000; Meyers *et al.*, 2003). The selection process is aimed at rearing egg lots from mating pairs with undetectable or very low levels of *R. salmoninarum*. Losses from BKD among the progeny of parents with very low levels of *R. salmoninarum* have been less than those among the progeny of parents with very high infection levels. Recent genetic analysis suggests that selective culling of female broodstock with high antigen titres will not affect the resistance of progeny (Hard *et al.*, 2006). As indicated earlier, broodstock segregation is unlikely to eliminate BKD if the water source is populated with feral fish infected with the organism. Because the broodstock used for commercial fish farming should be free of the kidney disease bacterium, it may be necessary to repopulate a contaminated facility with broodfish from a BKD-free population. Regular fallowing, particularly of marine sites, has helped break the disease cycle (Bruno, 2004) and an aggressive broodstock culling programme in Norway has correlated with a dramatic drop in BKD in farmed Atlantic salmon (Wiens and Dale, 2009).

The Disease Agent

Historical background

The first report of BKD was in Atlantic salmon (Mackie *et al.*, 1933). It has also been referred to as Dee disease, white boil disease, salmonid kidney disease, or corynebacterial kidney disease. Belding and Merrill (1935) were the first in the USA to report a Gram-positive bacterium associated with necrotic kidney lesions of salmonids, but they were unable to culture the disease agent. Cultivation of the organism on cysteine–blood-enriched medium made possible the completion of Koch's postulates and identification of the aetiological agent as a slow-growing Gram-positive bacterium (Rucker *et al.*, 1954; Ordal and Earp, 1956). For additional information on historical aspects of bacterial kidney disease, readers are referred to reviews by Fryer and Sanders (1981), Klontz (1983) and Bullock and Herman (1988).

Current classification

Initially, Ordal and Earp (1956) classified the organism as a *Corynebacterium* sp. Smith (1964) concurred with this classification based on specific characteristics of the bacterium, such as aerobic growth, lack of endospores, production of metachromatic granules, reproduction by binary fission and pathogenic capability. Young and Chapman (1978), however, were unable to find metachromatic granules or the post-fission snapping process associated with corynebacteria. Furthermore, the absence of mycolic acids (Goodfellow *et al.*, 1976; Fryer and Sanders, 1981), the presence of lysine in the peptidoglycan instead of *m*-diaminopimelic acid (Sanders and Fryer, 1980) and the difference in lipid composition (Collins, 1982; Embley *et al.*, 1983) do not support the placement of the bacterium in the genus *Corynebacterium* or *Listeria* (Bullock *et al.*, 1974). Sanders and Fryer (1980) concluded that the organism belonged to a unique genus and named the pathogen *Renibacterium salmoninarum* (kidney bacterium of salmon). Molecular cataloguing and sequencing of the 16S ribosomal ribonucleic acid (RNA) from *R. salmoninarum* (Stackebrandt *et al.*, 1988; Gutenberger *et al.*, 1991) and recalculation of the guanine plus cytosine (G+C) content (Banner *et al.*, 1991) supported the placing of the organism as a member of the high

G+C content, Gram-positive, eubacterial subdivision of the actinomycetes. Recently, the whole genome sequence (strain ATCC 33209) was determined (Wiens *et al.*, 2008) and the G+C content of 56.3% in concordance with previous estimates (Banner *et al.*, 1991) places the organism as a member of the high G+C group of Gram-positive bacteria. The genome contains 46 tRNAs and two identical rRNA operons. Comparisons of the 16S rRNA sequences and the whole genome with sequenced microbes indicate that the closest known relatives belong to the genus *Arthrobacter*. Extended blocks of synteny, albeit with numerous rearrangements, were noted between *R. salmoninarum* and two sequenced *Arthrobacter* species (Wiens *et al.*, 2008). A striking difference of the *R. salmoninarum* genome is that it is 38–40% smaller than either *Arthrobacter* sp. (strain FB24) or *A. aurescens* TC1. Furthermore, the G+C content is 6–9% lower than either sequenced species of *Arthrobacter*. The presence of 80 copies of three different insertion sequence elements, high frequency of pseudogenes and limited DNA modification and repair capabilities are consistent with the hypothesis that *R. salmoninarum* has undergone genome reduction in the process of adaptation from an environmental soil niche to a salmonid host (Wiens *et al.*, 2008). As yet, additional species have not been described for this genus, nor have subspecies been identified. It is of interest that non-pathogenic *Arthrobacter* have been isolated from Chinook salmon (*O. tshawytscha*) (Griffths *et al.*, 1998) and further genomic analyses of such strains may shed light on the evolutionary steps involved in host adaptation and pathogenesis.

Phenotypic and biochemical characteristics

R. salmoninarum is a small (0.3–1.5 μm × 0.1–1.0 μm), non-motile, non-acid-fast, non-spore-forming, strongly Gram-positive rod that usually occurs in pairs (Sanders and Fryer, 1980). The biochemical properties and cell wall composition of *R. salmoninarum* isolates appear to be remarkably conserved (Bruno and Munro, 1986c; Fiedler and Draxl, 1986). Biochemical characteristics include an inability to produce acid from sugars (Sanders and Fryer, 1980), a cysteine requirement for growth (Ordal and Earp, 1956; Daly and Stevenson, 1985). Surprisingly, *R. salmoninarum* lacks apparent functional genes critical to the *de novo* synthesis of amino acids serine, glycine, cysteine, asparagine and methionine (Wiens *et al.*, 2008). Genome analyses also suggest core central metabolic pathways are functional, including glycolysis, pentose phosphate, tricarboxylic acid (Krebs') cycle, pyruvate cycle and anaplerotic reactions. *R. salmoninarum* may be able to utilize several sugars and polyols as the organism contains genes to import and utilize glucose, fructose, arabinose, gluconate, glycerol and citrate. It also appears to be able to utilize a variety of carbon substrates for energy, including pyruvate, lactate, succinate, malate, glycerol-3-phosphate, proline, butanoly-CoA and fatty acids. Fatty acid utilization is noteworthy as there is an absence of *fabA* and *fabZ* homologues, suggesting that *R. salmoninarum* is incapable of synthesizing both unsaturated and saturated fatty acids and, therefore, has to scavenge these compounds from the salmonid host. Confirmation of the defects in amino acid biosynthesis and fatty acid metabolism await biochemical analyses.

R. salmoninarum contains genes encoding enzymes for biosynthesis of the following co-factors: riboflavin, FAD, FMN, pyridoxine, pantothenate (including 4′-phosphopantetheine), coenzyme A, lipoate and menoquinone (Wiens *et al.*, 2008). In addition, genes for the biosynthesis and recycling of folate, essential for purine and thymidylate metabolism, are present in the genome. *R. salmoninarum* is known to produce catalase (Ordal and Earp, 1956) and the genome encodes enzymes that confer resistance to oxygen radicals including superoxide dismutase, peroxidases and thioredoxin peroxidase.

Protease (Ordal and Earp, 1956; Smith, 1964; Rockey *et al.*, 1991b), deoxyribonuclease (DNAse) and β-haemolytic activities against salmonid erythrocytes have been

described (Bruno and Munro, 1986c). Two specific haemolysins have been identified biochemically and one cloned by expression of *R. salmoninarum* genomic DNA fragments into *Escherichia coli* and plating on blood agar plates (Evenden *et al.*, 1993; Grayson *et al.*, 1995a,d, 2002). This gene was designated *hly* and encoded as open reading frame (ORF) locus RSal33209_3168 in the genome. This gene and protein sequence, as well as others referred to in this chapter, is available by searching the National Center for Biotechnology Information (NCBI) website database (http://www.ncbi.nlm.nih.gov/guide/) for nucleotide or gene 'RSal33209_3168'. The number 3168 is the consecutive gene number starting from the first gene located at the putative origin of replication. RSal33209_3168 encodes peptidase and metalloproteinase motifs and thus may function in the digestion of proteins following secretion from *R. salmoninarum*. Genome analysis also identified three candidate haemolysins encoded by ORFs RSal33209_0811, RSal33209_3195 and RSal33209_3047. Each candidate haemolysin has a clear homologue in *Arthrobacter* spp., indicating that *R. salmoninarum* did not acquire them horizontally from an unrelated bacterial species. Their role in virulence evolution and fish adaptation is unknown.

Growth is aerobic and optimal at 15–18°C. The generation time during the log phase of growth is approximately 24 h. The presence of only two ribosomal loci and the numerous pseudogenes within metabolic pathways may account for its slow growth.

Antigenic characteristics

R. salmoninarum isolates are antigenically homogeneous using polyclonal rabbit antisera. Bullock *et al.* (1974) demonstrated serological homogeneity using agglutination and precipitin reactions with sonic extracts of whole *R. salmoninarum* from ten isolates from the USA. Fiedler and Draxl (1986) identified a homogeneous, polymeric polysaccharide, possessing an approximate subunit weight of 10 kDa, which was immunogenic to rabbits. The polysaccharide was similar among 13 isolates from the USA, Canada and Europe. In addition, they identified a predominant, trypsin-sensitive protein present on the cell surface with an approximate molecular mass of 70 kDa. Getchell *et al.* (1985) characterized *R. salmoninarum* antigens, using a variety of techniques, including immunodiffusion, rocket immunoelectrophoresis and two-dimensional immunoelectrophoresis. Seven antigens (A–G) were common among seven isolates. The major extracellular component is a 57 kDa protein named antigen F, which is heat stable (100°C for 0.5 h). Cross-adsorption experiments with other *R. salmoninarum* isolates demonstrated that this antigen was the predominant cell-surface antigen on all isolates studied. Analysis of antigen F using immunoblotting has revealed that, in addition to the major 57 kDa band, there is a related minor 58 kDa band (Wiens and Kaattari, 1989; Daly and Stevenson, 1990). The production of monoclonal antibodies (MAbs) to the 57/58 kDa protein (also referred to as p57; Rockey *et al.*, 1991b; Wiens and Kaattari, 1991) identified two conserved epitopes, common to ten isolates from geographically disperse locations (Wiens and Kaattari, 1989).

Serotypes

Unique antigens particular to specific *R. salmoninarum* isolates have also been reported (Arakawa *et al.*, 1987). However, the composition of these antigens is unknown and the antibodies are no longer available. Two antigenic groups of *Renibacterium* have been reported (Bandin *et al.*, 1992), and there is antigenic variation in p57 (Wiens *et al.*, 2002). A Norwegian isolate (strain 684) lacked the MAb 4C11 epitope. Sequencing of two major soluble antigen (*msa*) genes encoding p57 identified a single Ala^{139} to Glu mutation in both genes. This suggests that some recombination must occur, at least between the *msa* genes. Similar analysis of additional strains from Norway demonstrated that 5/8 isolates contained this sequence variation and that 4C11 epitope positive and negative strains

overlapped at the same geographical locations (Wiens and Dale, 2009). P57 protein produced by strain 684 exhibited enhanced binding to Chinook salmon leucocytes, suggesting a possible advantage of this sequence variation (Wiens *et al.*, 2002). The complete nucleotide sequences of the *msa* genes from a greater number of isolates need to be determined to understand the frequency of sequence variation. Sequence variation in p57 may have implications for immunodiagnostic and molecular assays that detect the p57 protein or the *msa* genes.

Ultrastructure

The bacterium possesses a unique peptidoglycan and an unusual cell wall polysaccharide containing *N*-acetylfucosamine in addition to galactose, rhamnose and *N*-acetylglucosamine (Kusser and Fiedler, 1983). The chemical structure of the galactose-rich polysacccaharide was determined recently to be a heptasaccharide repeating unit with a backbone consisting of linear, alternating 3- and 6-linked β-D-galactofuranosyl residues and side chains consisting of a trisaccharide element containing *N*-acetyl-D-glucosamine, *N*-acetyl-L-fucosamine and D-rhamnose at the non-reducing end (Sorum *et al.*, 1998b). This polysaccharide comprises more than 60% of the dry weight of trypsinized cell wall preparations (Fiedler and Draxl, 1986) and may be a primary constituent of the capsule (Dubreuil *et al.*, 1990b).

The genome of *R. salmoninarum* encodes a set of proteins that form a capsular polysaccharide assembly pathway. These proteins include homologues of at least seven proteins encoded in *Staphylococcus* species capsular synthesis genetic clusters. However, in contrast to the *Staphylococcus* genes, the *R. salmoninarum* capsular synthesis genes are not present at a single locus in the genome. ORFs RSal33209_1611 and RSal33209_1612 are homologous to *cap5G* and *cap5H* but are located in a different genomic region from other genes involved in the putative capsular synthesis pathway. These genes share no significant identity with sequences from *Arthrobacter* but are adjacent to genes involved in extracellular carbohydrate synthesis and transport (RSal33209_1613 through RSal33209_1618), which have homologues with matching gene order in both *Arthrobacter* spp. The regions encoding capsular synthesis proteins suggest that there have been multiple mechanisms of evolutionary divergence from *Arthrobacter* spp. First, four ORFs, RSal33209_1436 to RSal33209_1439, are located in a region of the chromosome that has synteny with regions in both *Arthrobacter* species. Only one of the candidate capsular synthesis ORFs (RSal33209_1436) is shared with one of the *Arthrobacter* species (strain TC1). The other candidate capsular synthesis genes were not found in the sequenced *Arthrobacter* spp., even though each genome had genes for extracellular polysaccharide synthesis at the same locus. It is possible that this region has been a locus of divergence as *R. salmoninarum* and *Arthrobacter* spp. occupy different environmental niches. A second locus, ORFs RSal 33209_1338 to RSal33209_1342, may represent a genomic island acquired by *R. salmoninarum* via a DNA horizontal exchange with another bacterial species. These ORFs encode proteins that can be placed in capsular synthesis pathways, have low G+C contents (48–51%) compared with the general *R. salmoninarum* genome (56.3%), and are flanked by complete and partial IS994 insertion elements. Therefore, we speculate that *R. salmoninarum* may have lost selected pathways associated with environmental exopolysaccharide synthesis, modified existing genes encoding exopolysaccharide synthesis proteins and gained a pathway associated with synthesis of a protective capsule (Wiens *et al.*, 2008). Gene products encoded by RSal33209_1750 and RSal 33209_2984 are similar to enzymes involved in UDP-glucose metabolism, and homologues of these gene products participate in capsular synthesis in a diverse collection of bacteria (Dillard and Yother, 1994; O'Riordan and Lee, 2004). These gene products also share sequence identity with proteins that participate in carbohydrate

metabolism in many different organisms, and these genes are present in the *Arthrobacter* sp. strain FB24 genome. As with our other *in silico* predictions based on metabolic reconstruction from the genome sequence, genetic analyses and biochemical isolation characterization are required to confirm the proposed capsule genes and link them to structures determined from biochemical analyses.

Transmission

There is evidence that the disease is transmitted both horizontally and vertically. Horizontal transmission by co-habitation is demonstrated under laboratory conditions (Bell *et al.*, 1984; Murray *et al.*, 1992); however, the route of entry is not clear. Infection via contaminated water may be one route of transmission. Viable *R. salmoninarum* is demonstrated in fresh water and salt water using FAT (fluorescent antibody technique) and bacterial culture (Austin and Rayment, 1985; McKibben and Pascho, 1999). The disease can also be transmitted via feeding infected adult viscera to juvenile fish (Wood and Wallis, 1955), leading to the discontinuance of feeding raw salmon products to juvenile fish (Fryer and Sanders, 1981). A natural source of infective material may be faeces of clinically infected or carrier fish. Oral intubation of infected faecal material, but not autoclaved material, transmits the microorganism and results in clinical disease (Balfry *et al.*, 1996). The ingestion of faecal material may contribute significantly to the horizontal transmission of *R. salmoninarum* among salmonids reared in seawater net pens (Balfry *et al.*, 1996). In an *in vitro* gill culture model, bacterial attachment and uptake are not observed, suggesting this may not be a route of infection (McIntosh *et al.*, 2000). However, bath challenge induces iNOS expression in gill tissue, indicating *Renibacterium*-responsive cells are present in gill tissue (Campos-Perez *et al.*, 2000).

R. salmoninarum is unusual among bacterial pathogens as it is transmitted to offspring via the egg. Allison (1958) and Bullock *et al.* (1978) were the first to report circumstantial evidence that gametes from infected adults, transferred to historically disease-free locations, resulted in clinically infected progeny. The bacterium has been identified on both the surface and the inside of eggs of a coho salmon infected naturally with 4×10^9 colony-forming units (CFU) *R. salmoninarum*/ml of ovarian fluid (Evelyn *et al.*, 1984). The bacterium was cultured from 15.1% of surface-disinfected eggs and observed within the yolk of sectioned eggs. Under laboratory conditions, infection of unfertilized steelhead (*O. mykiss*), coho (*O. kisutch*) and Chinook salmon eggs by immersion challenge has only been accomplished using high numbers of *R. salmoninarum* (1.4×10^9, 1.3×10^{12} and 1.7×10^5, respectively) (Evelyn *et al.*, 1986a). Under these conditions, only 1–5.5% of the experimentally exposed eggs contained viable *R. salmoninarum* (Evelyn *et al.*, 1986a; Lee and Evelyn, 1989). It is not clear why only low percentages are infected or how the bacterium enters eggs. Evelyn *et al.* (1986a) postulated that eggs in nature became infected after ovulation while they were in contact with coelomic fluid. However, Bruno and Munro (1986b) observed the presence of *R. salmoninarum* in tissue sections of maturing oogonia of experimentally infected trout. This suggests that egg infection may result directly from ovarian tissue prior to ovulation. Current evidence suggests that intraovum infection results in progeny with low levels of infection. Lee and Evelyn (1989) found that smolts reared from externally disinfected eggs from infected adults, but not uninfected adults, had subclinical infections. Low levels of *R. salmoninarum* (23–113 cfu/ml in adult ovarian fluid) were associated with a 1–2% infection in smolts and subclinical disease. Unfortunately, bacterial cells from FAT-positive smolts were not cultured or enumerated. The mechanism(s) of intraovum transmission and the contribution of vertically transmitted bacterium to overt disease warrant further investigation, as this is an uncommon mode of bacterial transmission. Furthermore, the availability of genomic markers now allows examination of whether the

strain in the parent is identical to that in the offspring.

Geographic Distribution and Host Range

BKD is common in cultured salmonid stocks in North America and its distribution closely mirrors cool and coldwater culture of salmonids (Fig. 9.1). *R. salmoninarum* is present in Continental Europe, Japan (Fryer and Sanders, 1981), South America (Sanders and Barros, 1986), Scotland (Bruno, 1986c) and Scandinavia (Gudmundsdóttir *et al*., 1993; Lorenzen *et al*., 1997; Jónsdóttir *et al*., 1998). Significant populations of free-ranging fish also harbour *R. salmoninarum* (Pippy, 1969; Evelyn *et al*., 1973; Ellis *et al*., 1978; Mitchum *et al*., 1979; Paterson *et al*., 1979, 1981b; Banner *et al*., 1986; Souter *et al*., 1987; Elliott and Pascho, 1991; Sanders *et al*., 1992; Meyers *et al*., 1993). BKD has not been reported in Ireland, Australia, New Zealand or the former Soviet Union (Bruno, 2004).

Animals affected, hosts and risk factors

Salmonids of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* appear to be the primary hosts of *R. salmoninarum* (reviewed in Fryer and Sanders, 1981). Natural infections have also been documented in *Hucho hucho* (Danube salmon; Pfeil-Putzien *et al*., 1985), *Thymallus thymallus* (grayling; Kettler *et al*., 1986), *Plecoglossus altivelis* (ayu; Nagai and Iida, 2002) and *Coregonus lavaretus* (whitefish; Rimaila-Parnanen, 2002). Experimental infection and mortality have been induced in a non-salmonid species, *Anoplopoma fimbria* (sablefish; Bell *et al*., 1990), and *Culpea harengus pallasi* (Pacific herring; Evelyn, 1993), but not in *Cyprinus carpio* (carp; Sakai *et al*., 1989b) or *Lampetra tridentata* (lamprey; Bell and Traxler, 1986).

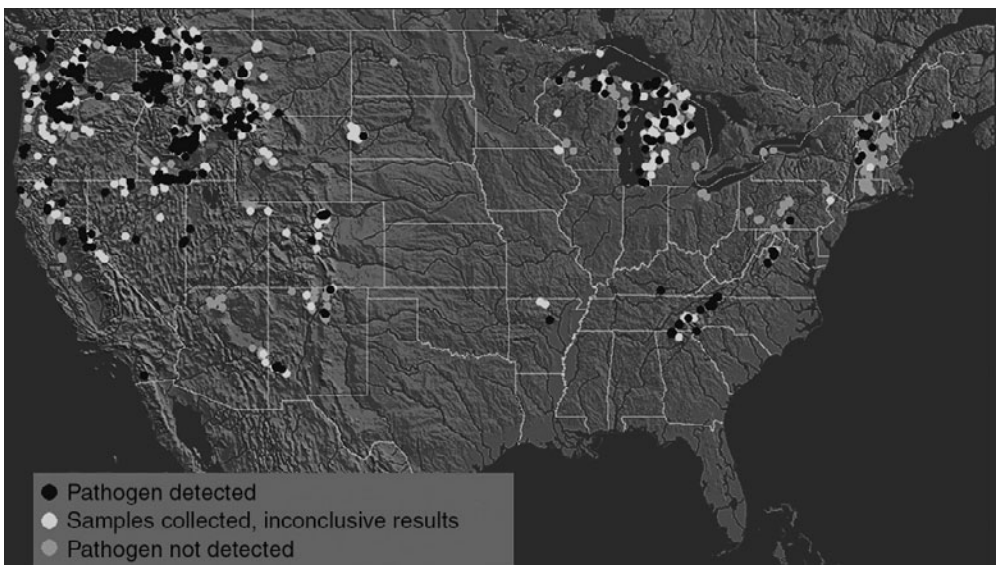


Fig. 9.1. Prevalence of *Renibacterium salmoninarum* in the USA. Image is from the Wild Fish Health Database (http://www.esg.montana.edu/nfhdb/) created by a search for 'Salmonide' and '*R. salmoninarum*'. The pathogen was detected in 799 samples from a total of 2963 samples obtained between 1981 and 2008. *R. salmoninarum* is most prevalent in the Pacific North-west and Great Lakes regions.

Sakai and Kobayashi (1992) found *R. salmoninarum* antigen in *Patinopecten yessoensis* (scallops), *Platycephalus indicus* (flathead) and *Cottus japonicus* (Japanese sculpin) sampled near a coho salmon saltwater net-pen facility. However, the importance of these species as reservoirs of infection is unclear, as viable *R. salmoninarum* could not be cultured. Efforts to identify *R. salmoninarum* in mussels (*Mytilus edulis*) and non-salmonid finfish which reside in and around seawater net pens in the Pacific North-west have not been successful (Paclibare *et al.*, 1994; Kent *et al.*, 1998). In addition, three species of freshwater bivalves challenged with *R. salmoninarum* failed to show infection or transmit *R. salmoninarum* to rainbow trout by cohabitation (Starliper and Morrison, 2000). These studies collectively indicate that *R. salmoninarum* is highly adapted for infection and persistence in salmonids.

Economic Importance of the Disease

The precise magnitude of the worldwide economic losses due to BKD is unknown, but is likely to be considerable. In the Pacific North-west, BKD is one of the most devastating bacterial diseases affecting wild and propagated anadromous salmonid stocks. It is especially problematic, as salmon are afflicted with the disease in both freshwater and salt-water life stages (Earp *et al.*, 1953; Fryer and Sanders, 1981; Banner *et al.*, 1983). BKD is considered to be one of the most significant causes of mortality for farmed Chinook and coho salmon (Kent, 1992) and has been implicated in the decline of the Lake Michigan Chinook salmon fishery in the late 1980s (Holey *et al.*, 1998). BKD is a high priority to the Atlantic salmon industry on the east coast of Canada (Griffiths *et al.*, 1998), although these fish are more resistant than Pacific salmon species (Kent, 1992). In Norway, the mortality caused by BKD is usually low, but cumulative losses as high as 70% have occurred at some locations (Dale *et al.*, 1997), causing millions of dollars in losses. Since the early 1990s, losses due to BKD have been reduced greatly, coinciding with increased biosecurity measures and culling programmes (Olsen *et al.*, 2007; Wiens and Dale, 2009).

Environmental Impact

Much research has focused on the impact of BKD on hatchery and wild spring/summer Chinook salmon in the Columbia River system (Bullock and Wolf, 1986; Warren, 1991; Williams, 2001). Extensive surveys of outmigrating smolts from the Columbia River system identified high percentages of *R. salmoninarum* antigen-positive fish (Maule *et al.*, 1996; Elliott *et al.*, 1997; VanderKooi and Maule, 1999). Although most fish had low levels of infection, in some tributary systems the majority of fish had medium to high infection levels (Maule *et al.*, 1996; Elliott *et al.*, 1997). Under experimental conditions, fish with medium to high levels of infection displayed increased vulnerability to predation (Mesa *et al.*, 1998), increased susceptibility to the effects of dissolved gas supersaturation (Weiland *et al.*, 1999), reduced feeding (Pirhonen *et al.*, 2000) and impaired saltwater adaptation (Moles, 1997; Mesa *et al.*, 1999). These findings, in conjunction with the high prevalence of *R. salmoninarum*, suggest that BKD may be an important factor contributing to poor smolt survival and low percentages of returning adult fish (Raymond, 1988; Williams, 2001). In addition to impacts on salmonid stocks in the USA, BKD is thought to have been transferred to Japan in the early 1970s with imports of coho salmon eggs and subsequently spread to the indigenous, and highly valued, cherry salmon (*O. masou*) (Scientific Committee on Animal Health and Welfare, 1999). Efforts to eradicate this disease in Japan have not been successful. The pathogen is present in Chile, correlating with the importation of Pacific salmonid species (Sanders and Barros, 1986).

The effects of BKD on endangered salmonid stocks in western North America

are a significant concern. The disease has emerged as the most prevalent and problematic fish health concern, limiting captive broodstock programmes being used to restore threatened Chinook and sockeye salmon populations from Idaho, Washington and Oregon (Flagg and Mahnken, 1995; Hoffnagle *et al.*, 2003, 2009). Affected Chinook salmon include populations from the Grande Ronde and Imnaha basins that are a portion of the Endangered Species Act-listed Snake River evolutionary significant unit (Federal Register 1992, Volume 57, Number 78) (Hoffnagle *et al.*, 2009).

Diagnostic Methods

Crucial to the success of any BKD control programme is the application of reliable diagnostic methods that detect low *R. salmoninarum* levels in a variety of sample types. For that reason, fish health specialists and researchers have long been interested in developing methods for rapid and reliable detection of *R. salmoninarum* infections (Pascho *et al.*, 1987, 1998; Sakai *et al.*, 1989a; Gudmundsdóttir *et al.*, 1993; White *et al.*, 1995). Nevertheless, no single ideal diagnostic test has yet been developed for the evaluation of multiple sample types for BKD (reviewed in Pascho *et al.*, 2002). BKD was formerly an OIE listed disease but was removed from the 12th edition as a notifiable disease (http://www.oie.int/eng/maladies/en_classification2009.htm?e1d7) in 2009. Standardized diagnostic procedures for detection of *R. salmoninarum* are described on the OIE website.

Clinical

Historically, two general strategies for diagnosis of infection have been pursued: (i) direct detection of *R. salmoninarum* or antigens using culture methods, fluorescent antibody staining, enzyme-linked immunosorbent assay (ELISA) or PCR; and (ii) detection of fish antibodies to *R. salmoninarum*. Of the two, direct detection of *R. salmoninarum* and its products is the most widely used. Early efforts relied on the observation of Gram-positive diplobacilli and the presence of clinical signs (Earp *et al.*, 1953; Bell, 1961; Pippy, 1969). Heavily infected fish can display a variety of signs including exophthalmia, erratic swimming behaviour, superficial blebs of the skin and muscle lesions (Smith, 1964; Fryer and Sanders, 1981; Bullock and Herman, 1988). The use of Gram stain is limited because of its low sensitivity ($1\times10^{7-9}$ bacterial cells/g tissue) (Bullock *et al.*, 1980; Pascho *et al.*, 1987; Sakai *et al.*, 1987), and the presence of melanin granules in kidney tissue can obscure low numbers of organisms. A periodic acid-Schiff stain can locate *R. salmoninarum* in tissue sections; however, it is not a specific stain (Bruno and Munro, 1982).

Culture

Culture of *R. salmoninarum* from fish tissues, followed by serological identification or PCR, is the definitive test (Fryer and Sanders, 1981; OIE, 2003). Several different media support bacterium growth. Originally, Ordal and Earp (1956) used a complex blood medium supplemented with cysteine (kidney disease medium 1 [KDM–1]). Subsequently, Evelyn (1977) devised a kidney disease medium containing 1% (w/v) peptone, 0.05% (w/v) yeast extract, 0.1% (w/v) cysteine and 20% serum (KDM-2), which allowed the primary isolation of bacteria from fish tissues. Charcoal can replace serum components (Daly and Stevenson, 1985; Daly, 1989) and specific growth components have been described (Embley *et al.*, 1982; Shieh, 1988a). Heavy inocula of 'nurse culture' in the centre of the Petri dish enhance primary isolation (Evelyn *et al.*, 1989) or the addition of 1.5–5% spent media to culture plates (Evelyn *et al.*, 1990; Teska, 1994). Enhanced growth under these conditions is due presumably to the action of a diffusible factor hypothesized either to inactivate a toxic component in the medium or to stimulate growth of the bacterium. The nature of this component is unknown, but the analysis of the genome has identified

two resuscitation-promoting factors. Resuscitation-promoting factors are a family of proteins that are common in high G+C-content bacteria and are involved in reactivation of bacterial growth from a dormant state. *R. salmoninarum* contains two genes, RSal33209_2532 and RSal33209_2995, that have a resuscitation-promoting factor domain. These genes encode predicted proteins consisting of 220 and 384 amino acids, respectively. Both proteins have predicted signal peptide sequences, suggesting export.

Regardless of the medium used, primary isolation of *R. salmoninarum* is difficult and time-consuming. Isolation of colonies from a highly infected fish takes from approximately 2 weeks (Evelyn, 1977) to as long as 19 weeks for subclinical cases (Benediktsdóttir *et al.*, 1991). One problem is contamination with heterotrophic bacteria. The bacteria grow much faster than *R. salmoninarum* and tend to overgrow Petri dishes. Addition of four antimicrobials (0.005% w/v cycloheximide, 0.00125% w/v D-cycloserine, 0.0025% polymyxin B sulfate and 0.00025% oxolinic acid) to KDM-2 allows the selective isolation of *R. salmoninarum* (Austin *et al.*, 1983). When using culture techniques, care in interpreting the results from kidney is required, as disrupted tissue is inhibitory to *in vitro R. salmoninarum* growth (Evelyn *et al.*, 1981; Daly and Stevenson, 1988). Additionally, *R. salmoninarum* is extremely sensitive to some media components, for example, the bacteria grow poorly in some batches of peptone (Evelyn and Prosperi-Porta, 1989). In general, the fastidious culture requirements, slow growth of *R. salmoninarum* and common overgrowth of Petri dishes with contaminating organisms limit the usefulness of culture as a rapid method to screen large numbers of fish.

Immunodiagnosis

Since culture of *R. salmoninarum* is difficult and Gram stain is insensitive, a number of immunodiagnostic assays have been developed. Bullock and Stuckey (1975) first described the direct fluorescent antibody technique (FAT) to visualize bacterial cells directly. The FAT is more sensitive than the Gram stain and can detect subclinical infections (Bullock and Stuckey, 1975; Laidler, 1980). There are several methods to quantify *R. salmoninarum* employing fluorescent antibodies, including a subjective scoring of fluorescence intensity (1+ to 4+) of tissue smears (Bullock *et al.*, 1980) and a membrane FAT procedure. In the latter procedure, bacteria are immobilized on filter-paper grids and titres expressed as cells per unit of tissue or ovarian fluid (Elliott and Barila, 1987). This assay detects less than 10^2 bacterial cells/ml of coelomic fluid (Elliott and Barila, 1987) and has a similar sensitivity when used to enumerate *R. salmoninarum* cells in tissues (Lee, 1989). While the FAT is a very sensitive test for *R. salmoninarum*, it suffers from being tedious to perform, labour-intensive when large numbers of samples are examined and less accurate with low levels of *R. salmoninarum* (Armstrong *et al.*, 1989).

An alternative method of diagnosis is the detection of soluble antigens. Soluble antigens were detected first from the kidney, liver, spleen and blood of infected fish using the immunodiffusion technique (Chen *et al.*, 1974). Kidney tissue contains higher concentrations of these antigens and is the most useful for diagnosis. A 100% correlation (n=30) was observed between positive precipitin reactions, the presence of clinical signs and a positive Gram stain (Kimura *et al.*, 1978). A major limitation, however, is the inability of the immunodiffusion technique to detect low levels of *R. salmoninarum* (Fryer and Sanders, 1981; Cipriano *et al.*, 1985; Sakai *et al.*, 1989a). Counter-immunoelectrophoresis is more sensitive than immunodiffusion or bacterial culture (Cipriano *et al.*, 1985), but can be variable (Pascho *et al.*, 1987). Detection of subclinically infected fish was accomplished by Kimura and Yoshimizu (1981) using the staphylococcal coagglutination technique. This qualitative test requires 1.5 h to complete and is as sensitive as the FAT (Sakai *et al.*, 1987).

The most widely used assays for rapid detection of *R. salmoninarum* antigen in

large surveys are the dot-blot and the ELISA. The dot-blot technique detects antigen qualitatively after samples are coated on to nitrocellulose (Sakai *et al.*, 1987). ELISAs are more advantageous, as they allow precise quantitative detection of antigen (Dixon, 1987; Pascho and Mulcahy, 1987; Turaga *et al.*, 1987b; Hsu and Bowser, 1991; Rockey *et al.*, 1991b; Gudmundsdóttir *et al.*, 1993; Olea *et al.*, 1993). The ELISA developed by Dixon (1987) detects antigen rapidly in 0.5 h but is unable to detect low levels associated with subclinically infected fish. Other assays (Pascho and Mulcahy, 1987; Turaga *et al.*, 1987b) use longer incubation periods and detect antigens in amounts as low as 2–20 ng and 10 ng, respectively. Rockey *et al.* (1991a) developed an antigen-capture, MAb-based ELISA that was specific for two epitopes present on p57. ELISA technology possesses equivalent or greater sensitivity than either culture or the FAT (Pascho *et al.*, 1987; Rockey *et al.*, 1991a).

Indirect detection of *R. salmoninarum* infection by the identification of specific salmonid antibodies has not met with widespread success. Salmonids produce agglutinating antibodies to *R. salmoninarum* (Evelyn, 1971); however, antibody levels do not seem to correlate with the level of infection. Banowetz (1974) assayed 207 yearling coho salmon from a population during an epizootic of BKD and found that fish agglutinin titres equal or higher than 1:128 generally had no detectable bacteria. Conversely, *R. salmoninarum*-positive fish had low agglutinin titres. Similar findings were reported by Bruno (1987), who found that serum agglutinins were not detected in heavily infected Atlantic salmon smolts, but that the titre increased prior to the end of an epizootic. The variable levels of serum agglutinins in apparently healthy fish (Evelyn *et al.*, 1981; Paterson *et al.*, 1981a; Bruno, 1987) and the low levels of detectable agglutinins in infected fish suggest that serum agglutination assays are of limited value for monitoring fish health (Banowetz, 1974; Bruno, 1987; Jansson and Ljungberg, 1998). However, since recovering fish produce a humoral response to *R. salmoninarum*, detection of seropositive fish using agglutination, ELISA (Bartholomew *et al.*, 1991; Jansson *et al.*, 2003) or electroimmunotransfer blot assays (Olivier *et al.*, 1992) may be useful methods for monitoring disease progression under experimental conditions.

Antigenic cross-reactivity

The development of highly sensitive ELISA techniques has identified many more antigen-positive fish than previously had been identified using FAT or culture techniques (Gudmundsdóttir *et al.*, 1993; Meyers *et al.*, 1993). The antigen-positive samples not confirmed using another technique are interpreted with caution. It should not be assumed that these fish would, at some point, develop clinical BKD, as the fish might harbour cross-reactive substances, which would interfere with the ELISA, and/or antigen might be present but the bacteria might be non-viable.

The sensitivity and specificity of the ELISA relies heavily on the quality of antisera used. Polyclonal antibodies and MAbs can cross-react with other organisms. Cross-reactive Gram-positive, Gram-negative and Gram-undetermined organisms have been identified using FAT (Bullock *et al.*, 1980; Evelyn *et al.*, 1981; Austin *et al.*, 1985; Yoshimizu *et al.*, 1987; Brown *et al.*, 1995; Teska *et al.*, 1995) and ELISA (Brown *et al.*, 1995; Bandin *et al.*, 1996). The molecular basis for the cross-reactivity is unidentified but may include common carbohydrate molecules, such as galactose (Fiedler and Draxl, 1986) or heat-shock proteins (Wood *et al.*, 1995). Dixon (1987) reported that pre-adsorption of polyclonal antisera with *Rothia dentocariosa* and *Bacillus sphaericus* increased specificity of the ELISA. In addition to cross-reactivity with microorganisms, cross-reactivity of polyclonal antisera has been observed in the ELISA with feather meal components present in certain commercial diets (Pascho *et al.*, 1991a) and with fish serum components (Turaga *et al.*, 1987a). Thus, affinity purification of polyclonal antisera is probably necessary to ensure adequate specificity.

The use of MAbs may increase the specificity of the immunoassay, as these antibodies recognize precise, selected epitopes. Judicious choice of MAbs is necessary, as Arakawa *et al.* (1987) identified a MAb that recognized a cross-reactive determinant on *R. salmoninarum* and three other Gram-positive organisms.

Since cross-reactive antigens exist, confirmation of ELISA results requires the use of independent assays. Western blotting is one technique for the confirmation of *R. salmoninarum* p57 in ELISA-positive samples, since molecular mass and immunoreactivity can be used to distinguish p57 (Wiens *et al.*, 1990). A disadvantage of this technique is that it is three- to fourfold less sensitive than the monoclonal-based ELISA (Wiens, 1992). Alternatively, PCR is a sensitive method for detection of the *msa* gene encoding p57. However, even if the p57 protein or gene is confirmed, the presence of viable organisms is still in question. Therefore, it is recommended that, if putative *R. salmoninarum*-free stocks are identified as ELISA-positive, Western blot or PCR, in conjunction with culture techniques, be used to confirm the presence of p57 and viable *R. salmoninarum*. Using this type of combined approach for detecting *R. salmoninarum*, Griffiths *et al.* (1996) found that incubation of ovarian fluid cellular debris in selective kidney disease medium (SKDM) broth, followed by Western blotting, increased the total numbers of positive samples by 32% over SKDM agar culture or indirect FAT (IFAT).

Molecular probes/techniques

The availability of gene sequences from *R. salmoninarum* has facilitated the development of nucleic acid-based diagnostic assays. These assays are generally highly specific and can be performed rapidly (Pascho *et al.*, 2002). Several nucleic acid hybridization approaches have been reported (Etchegaray *et al.*, 1991; Mattsson *et al.*, 1993; Leon *et al.*, 1994; Hariharan *et al.*, 1995); however, there is little information on the relative sensitivities of these assays compared with traditional diagnostic methods (Pascho *et al.*, 2002). Nucleic acid technologies that amplify a target sequence using the polymerase chain reaction (PCR) are generally more sensitive than DNA probes. Both PCR and reverse-transcription PCR assays have been developed to measure either the 16s RNA gene (Magnusson *et al.*, 1994; Rhodes *et al.*, 1998) or the gene encoding p57 (Brown *et al.*, 1994, 1995; McIntosh *et al.*, 1996; Miriam *et al.*, 1997; Chase and Pascho, 1998; Cook and Lynch, 1999). Most PCR assays that detect *R. salmoninarum* are very sensitive for detecting the pathogen in a variety of tissue types. Nested PCR amplification of p57 is equal to or greater in sensitivity than detection by ELISA or FAT (Chase and Pascho, 1998). PCR is also equal to or greater than that of bacteriological culture using kidney tissue (McIntosh *et al.*, 1996; Miriam *et al.*, 1997; Cook and Lynch, 1999) or coelomic fluid (Miriam *et al.*, 1997). PCR may be especially useful for detecting *R. salmoninarum* in ovarian fluid (Miriam *et al.*, 1997; Pascho *et al.*, 1998) as both polyclonal- and monoclonal-based ELISA do not detect low bacterial cell concentrations consistently (Wiens, 1992; Griffiths *et al.*, 1996; Pascho *et al.*, 1998). Bruno *et al.* (2007) used a non-destructive real-time qPCR assay for whole blood to detect a 79 bp fragment of the *R. salmoninarum* 57 kDa surface protein. Overall, a limitation of genomic DNA amplification, similar to ELISA and FAT, is that the viability of the pathogen is unknown. Reverse-transcription PCR, however, requires the presence of bacteria RNA that degrades rapidly once a bacterium is non-viable. Cook and Lynch (1999) demonstrated that RT-PCR detection of the *msa* genes required viable *R. salmoninarum* and loss of bacterial cell viability by rifampin or erythromycin treatment paralleling message reduction. This assay may be useful for distinguishing the continued presence of viable bacteria, particularly in samples from broodstock treated with antibiotics prior to spawning. Currently, PCR assays are impractical for large-scale broodstock screening programmes due to equipment capacity limitations, the manipulations required to

extract samples and concerns about cross-contamination (Pascho *et al.*, 2002). However, PCR is an accepted confirmatory test for the ELISA or FAT. The ability to detect and distinguish multiple pathogens at one time (Nilsson and Strom, 2002) and the ability to determine message quantities precisely using real-time PCR (Powell *et al.*, 2005; Chase *et al.*, 2006; Rhodes *et al.*, 2006; Suzuki and Sakai, 2007; Jansson *et al.*, 2008; Saleh *et al.*, 2008; Halaihel *et al.*, 2009) increased the utility of these molecular-based assays.

Genome Structure and Transcription

Genome

The *R. salmoninarum* genome sequence of strain ATCC 33209 is a single circular chromosome of 3,155,250 bases accessed as GenBank number NC_010168 (Wiens *et al.*, 2008). The ATCC 33209 strain lacked integrated phage or associated plasmids. Other strains may contain plasmid(s), as variable numbers of the *msa* gene have been identified using Southern blotting (Rhodes *et al.*, 2004a; Wiens and Dale, 2009) and several large plasmids have been identified in *Arthrobacter* spp. (Mongodin *et al.*, 2006).

Genes and proteins

The genome encoded 2777 protein-coding sequences and manual annotation identified 730 'partial' protein-coding sequences that represented putative pseudogenes (Wiens *et al.*, 2008). Of the partial gene sequences, 360 were disrupted by frameshifts, 208 by point mutations and 162 by uncharacterized changes including insertion sequences and putative deletions. The precise number of pseudogenes is difficult to determine due to the large number of hypothetical genes that appear to be disrupted and, therefore, the number should serve as an estimate of total disrupted genes. The membrane transporters category of genes contains the highest percentage of disrupted ORFs (43%), and 31% of fatty acid/phospholipids metabolism ORFs are partials. In contrast, the lowest number of partial ORFs is in protein synthesis, transcription and mobile genetic elements, with each class being below 8%.

Protein expression

R. salmoninarum contains a complete Sec-dependent secretion pathway, with the exception of a YajC homologue (protein translocase). Four homologues of signal peptidase I (RSal33209_1086, RSal33209_1087, RSal33209_1255 and RSal33209_2979) and one homologue of signal peptidase II (lipoprotein signal peptidase; RSal33209_2485) are present. A SignalP and TmPred search demonstrated the presence of at least 444 ORF products having a typical Sec secretion leader peptide. However, this number should be approached with caution as two different ORF calling programmes used (IG and TIGR) exhibited quite different gene predictions of about 30% of the ORF length (Wiens *et al.*, 2008). Difficulty in predicting ORF start sites in high G+C bacteria is well known and highlights the need for experimental verification using high throughput sequencing and/or proteomic approaches.

The *R. salmoninarum* genome contains an ORF encoding a sortase, a member of a group of cysteine transpeptidases in Gram-positive bacteria that promote covalent anchoring of proteins to the peptidoglycan surface of the cell envelope. The sortase (RSal33209_2896) is a member of subfamily 5 or class D of bacterial sortases and shares 65 and 62% identity with the sortases in *A. aurescens* TC1 and *Arthrobacter* sp. strain FB24, respectively. Using the tripartite pattern search method of Boekhorst *et al.* (2005), as well as tools such as Pfam, SignalP, TMpred and ProDom, seven ORFs containing the LAxTG motif have been identified and thus are potential sortase substrates, which are often involved in pathogen virulence. The candidate sortase substrates are RSal 33209_2105, RSal33209_3407, RSal33209_2525, RSal33209_3273, RSal33209_0619, RSal33209_1326 and RSal33209_ 0402; several are predicted to

be cell surface proteinases or adhesins. Two of the seven potential SrtD substrates are pseudogenes (RSal33209_0619 and RSal33209_1326). Three sortase substrates have *Arthrobacter* homologues (RSal33209_3407, RSal33209_1326 and RSal33209_0402), and three are unique to *R. salmoninarum* (RSal33209_2105, RSal33209_2525 and RSal33209_3273). The remaining putative sortase substrate (RSal33209_0619) shares sequence identity with candidate acid phosphatases in Gram-negative bacteria.

Recent drug discovery efforts have focused on sortases as a possible therapeutic target, and it is possible that such efforts will be important in preventing *R. salmoninarum* infection (Sudheesh *et al.*, 2007). Treatment of *R. salmoninarum* with sortase inhibitors decreases bacterial adherence to fibronectin and fish cells, suggesting that these proteins have a role in attachment (Sudheesh *et al.*, 2007). While *R. salmoninarum* SrtD is the only apparent active sortase, the presence of a degenerate *srtA* gene and at least one degenerate SrtA substrate with homologues in *Arthrobacter* may represent additional examples of specific genome decay in a pathway not needed for the intracellular lifestyle of *R. salmoninarum*.

Pathogenesis and Immunity

Disease progression

BKD often exhibits chronic pathology similar to mycobacterial diseases in mammals. Chronic inflammatory and granulomatous reactions characterize intracellular infection with associated tissue necrosis. In Pacific salmon, granulomas are diffuse with poorly defined borders, whereas the granulomas in Atlantic salmon are encapsulated with caseation in the centres (Kent, 1992). Histopathological changes occur after intraperitoneal injection of live or formalin-fixed *R. salmoninarum* into rainbow trout (Bruno, 1986b). Within 45 min of injection, live bacterial cells are in phagocytes of the kidney and spleen. After 4–6 days, *R. salmoninarum* is disseminated throughout these organs and, by 6–10 days, large numbers of *R. salmoninarum* are in blood monocytes and macrophages, where they appear to multiply. At 14 days, phagocytic cells containing *R. salmoninarum* are between myocardial bundles in the heart. After 28 days, *R. salmoninarum* are intracellular in endothelial cells lining the glomerular blood vessels and lumen of collecting ducts, but not within proximal tubules of the kidney. Colonization of kidney tissues and cell-mediated immunity to *R. salmoninarum* results in development of granulomatous lesions. CNS involvement is more common in farmed Atlantic salmon compared with Chinook salmon (Speare *et al.*, 1993; Speare, 1997). The reason for the different pathological presentation between species is unknown. Thymic infection can occur in experimentally infected coho salmon (Flano *et al.*, 1996a).

R. salmoninarum is a facultative intracellular pathogen. The bacterium appears to have an affinity for phagocytes, sinusoidal cells and reticular and barrier cells (Flano *et al.*, 1996b). The bacterium binds the C3b component of the complement pathway that enhances phagocytic uptake (Rose and Levine, 1992). In addition, *R. salmoninarum* p57 binds leucocytes that may aid attachment and invasion (Wiens and Kaattari, 1991). Following infection of macrophages *in vitro*, *R. salmoninarum* stimulates the respiratory burst (Campos-Perez *et al.*, 1997), which can be inhibitory for bacterial growth when macrophages are activated by cytokine simulation (Hardie *et al.*, 1996). Phagocytic cells often contain *R. salmoninarum* (Young and Chapman, 1978; Bruno, 1986b; Zhuo, 1990), although conclusive evidence of intracellular replication has been difficult to substantiate (Bandin *et al.*, 1993). The bacterium appears to escape from the macrophage phagosome into the cytoplasm, as observed using transmission electron microscopy (Gutenberger *et al.*, 1997). The histological observations combined with transmission electron microscopy (TEM) evidence suggest that *R. salmoninarum* is an invasive organism capable of causing a disseminated infection. Tissue culture models for studying the process of intracellular invasion

have been developed (McIntosh *et al.*, 1997) and an *R. salmoninarum* DNA fragment has been isolated which can confer internalization of *E. coli* into the Chinook salmon embryo cell line (CHSE-214) (Maulen *et al.*, 1996). Intracellular invasion is a common strategy of a number of pathogenic bacteria, thereby facilitating the access to nutrients and evasion from the immune system.

Pathology

Fish with severe *R. salmoninarum* infections may show no obvious external signs, or they may exhibit one or more of the following: lethargy; skin darkening; abdominal distension due to ascites; pale gills associated with anaemia; exophthalmia; haemorrhage around the vent; and cystic cavities in the skeletal muscle. Internal examination usually reveals the presence of focal to multifocal greyish-white nodular lesions in the kidney (Fig. 9.2a), and sometimes in the spleen and liver (Fig. 9.2b). In addition, there may be turbid fluid in the abdominal cavity, haemorrhages on the abdominal wall and in the viscera, and a diffuse white membranous layer (pseudomembrane) on one or more of the internal organs (Fryer and Sanders, 1981; Evelyn, 1993; Evenden *et al.*, 1993).

Infection changes a number of haematological and serum parameters in both experimentally and naturally infected fish. Circulating erythrocytes decrease 59–66% during infection (Bruno, 1986a,b). In addition, erythrocyte diameter decreases from 16.6 μm to 14.5 μm and erythrocyte sedimentation rate increases. Increased progression of disease correlates with decreased haematocrit, cholesterol, sodium and electrophoretically faster migrating serum proteins, as well as an increase in serum bilirubin, blood urea nitrogen and potassium concentrations (Hunn, 1964; Suzumoto *et al.*, 1977; Aldrin *et al.*, 1978; Bruno, 1986a; Turaga *et al.*, 1987a). No changes in small and large lymphocyte numbers occur, but there is a transitory increase in neutrophils, monocytes and thrombocytes after bacterial injection (Bruno and Munro, 1986a). Increased levels of p57 protein correlate with the severity of infection and a decrease in haematocrit (Turaga *et al.*, 1987b). High levels of p57 are found in experimentally and naturally infected fish (Turaga *et al.*, 1987b; Rockey *et al.*, 1991b), and humoral immunity to p57 has been hypothesized to result in immune complex

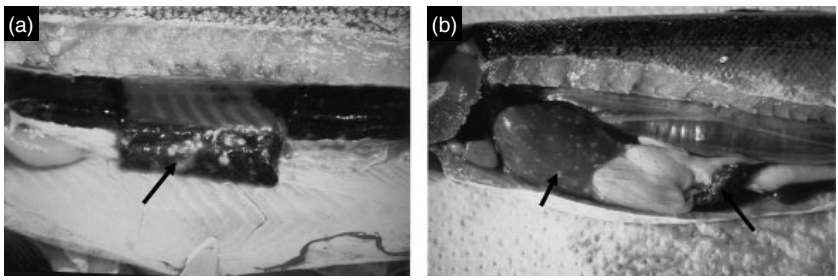


Fig. 9.2. (a) Section of the kidney of an infected coho salmon reared in a commercial net-pen salmon farm in Puget Sound, Washington State, USA. Note the granulomatous foci scattered thoughout the kidney (arrow). Lesions are typically found in naturally infected fish and fish infected experimentally with a low dose of *R. salmoninarum*. (b) Exposed midsection of an infected coho salmon. Arrows denote granulomatous lesions in the liver (left) and in the spleen (right). Photographs are courtesy of Dr J. Heidel, Department of Veterinary Medicine, Oregon State University, Oregon, and Dr R. Elston, Battelle Pacific Northwest Laboratories, Washington State.

formation and subsequent hypersensitivity reactions in the glomeruli of the kidney (Turaga, 1989; Kaattari and Piganelli, 1997).

Virulence factors

A number of enzymatic activities of *R. salmoninarum* have been identified which may contribute to virulence. These include haemolytic (Bruno and Munro, 1982), proteolytic (Smith, 1964; Bruno and Munro, 1986c; Rockey *et al.*, 1991b), exotoxin (Shieh, 1988b), catalase (Bruno and Munro, 1982), DNAse (Bruno and Munro, 1982) and iron reductase activities (Grayson *et al.*, 1995b). Several putative virulence genes have been cloned including haemolysin (*rsh*; Evenden *et al.*, 1990; Grayson *et al.*, 1995a), a zinc-metalloprotease (*hly*; Grayson *et al.*, 1995d) and a glucose kinase (Maulen *et al.*, 1996; Concha and Leon, 2000). In addition, a 50–100 nm capsule, a common virulence factor of other bacteria, has been observed using electron microscopy (Dubreuil *et al.*, 1990b). However, conclusive proof of the contribution of these enzymes, cloned genes, or the capsule to isolate virulence is not available.

Several investigators reported pathological changes in infected fish consistent with *in vivo* secretion of a toxin (Bruno, 1986a; Bell *et al.*, 1990). Bruno (1986a) observed an accumulation of erythrocytes in the spleen of experimentally infected rainbow trout and suggested that a toxin might be damaging erythrocytes, resulting in their sequestration within the spleen. Additionally, the lack of histologically detectable bacterial cells in the brain of experimentally infected sablefish that developed meningitis suggests the presence of a toxin (Bell *et al.*, 1990). Shieh (1988b) identified a putative exotoxin in *R. salmoninarum* culture supernatant, which at a dose of 160 µg was lethal for Atlantic salmon fingerlings (9–12 g). Unfortunately, a control extract was not prepared to rule out the possibility that the exotoxin was a component of the semi-defined medium (Shieh, 1988a). In contrast to the work of Shieh (1988a), other investigators have been unable to demonstrate toxicity with extracellular product (ECP) either by intraperitoneal injection of rainbow trout fingerlings or by addition of ECP to fish cell lines (Bandin *et al.*, 1991). We have not found *R. salmoninarum* ECP to exhibit cytotoxic effects when added to *in vitro* cultures of salmonid leucocytes as determined using trypan blue cell viability staining (Turaga *et al.*, 1987a; Wiens and Kaattari, 1991). These experiments, however, did not rule out the possibility of toxicity to a small set of leucocytes. The purification of extracellular components to homogeneity, as demonstrated using sodium docedyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), is formally required to identify exotoxin(s) produced by *R. salmoninarum*.

In bacterial species that are difficult to manipulate genetically, such as *Renibacterium* (Rhodes *et al.*, 2002), one strategy for the identification of putative virulence factors is to isolate spontaneous mutants with reduced virulence and to compare these with virulent isolates using biochemical or DNA analysis (Smith, 1989). Several spontaneous *R. salmoninarum* mutants have been identified which have reduced virulence by intraperitoneal challenge (Bruno, 1988; Griffiths *et al.*, 1998; Daly *et al.*, 2001). In rainbow trout, low-virulence isolates, MT238, MT239 and MT240, caused 8–18% total mortality compared with virulent isolates that produced 73–81% mortality (Bruno, 1988). Strain MT239 also has reduced virulence in Chinook salmon, a species highly susceptible to BKD (O'Farrell *et al.*, 2000). In rainbow trout, gross lesions are present in fish injected with virulent or low-virulence isolates. The low-virulence isolates were catalase positive and haemolytic against horse and sheep blood. Interestingly, the isolates with low virulence no longer possessed the typical *R. salmoninarum* trait of autoagglutination in culture, had reduced cell-surface hydrophobicity (Bruno, 1988) and lacked an amorphous layer associated with the cell surface (Senson and Stevenson, 1999). *R. salmoninarum* re-isolated from fish infected with these reduced-virulence

isolates did not revert to the original phenotype and were stable during an 18-month period. The absence of a saline-extractable 57 kDa protein was correlated with virulence reduction, suggesting that the cell-associated 57 kDa protein may be a virulence factor (Bruno, 1990). In support of this possibility, *R. salmoninarum* isolates containing three copies of the *msa* gene encoding p57 are more virulent than isolates containing two copies (Rhodes *et al.*, 2004a).

The 57/58-kilodalton protein (p57)

The most thoroughly characterized protein produced by *R. salmoninarum* is the 57/58 kDa protein (p57). This protein is an immunodominant antigen, which is both secreted and present on the bacterial cell surface (Getchell *et al.*, 1985; Turaga *et al.*, 1987b; Wiens and Kaattari, 1989). The protein can be either extracted by washing cells in an acidic pH (Daly and Stevenson, 1990) or concentrated from culture supernatant (Getchell *et al.*, 1985). When extracts are electrophoresed under reducing and denaturing conditions, a predominant 57 kDa band is apparent, as well as a minor 58 kDa band (Getchell *et al.*, 1985; Wiens and Kaattari, 1989; Daly and Stevenson, 1990). The gene coding p57 has been cloned and sequenced (*msa*, major soluble antigen) and it encodes a protein of 557 amino acids with a calculated *M*r of 57,190 (Chien *et al.*, 1992). Surprisingly, two copies of *msa* are present in most strains (O'Farrell and Strom, 1999; Wiens *et al.*, 2002) and, recently, strains have been identified containing at least three copies of *msa* (Rhodes *et al.*, 2004a; Wiens and Dale, 2009). Amino acids 1–26 encode a putative leader peptide sequence, which, after processing, results in a mature 54,505 kDa protein. The amino-terminal sequence derived from microsequencing p57 agrees with the predicted sequence of the protein starting at residue 27 (Radacovici and Dubreuil, 1991; Wiens and Kaattari, 1991; Chien *et al.*, 1992). It is unclear whether the 58 kDa protein represents the unprocessed protein containing the leader sequence or is the result of another type of modification of the mature protein. The 57 and 58 kDa proteins are not complexed by disulfide bonding, as the addition of β-mercaptoethanol does not change the electrophoretic migration (Daly and Stevenson, 1990). P57 contains two sets of sequence repeats: there are two direct A repeats, which are each 81 amino acids in length, and there are five B repeats, which are approximately 25 amino acids in length (Chien *et al.*, 1992; Wiens *et al.*, 1999). Interestingly, the A repeats contain a transcription factor immunoglobulin (TIG)-like domain which is found in the extracellular domain of members of the plexin protein family of adhesion-repulsion molecules (Wiens *et al.*, 2002). MAb 4D3 that binds to the A1 repeat also neutralizes p57 erythrocyte binding (Wiens and Owen, 2005). Leucocyte binding appears mediated by a region located N-terminal to the A1 repeat. The B repeats do not share significant sequence homology to other proteins.

It is unclear if p57 is post-translationally modified or if the protein assembles as part of a larger structure. Schiff staining for carbohydrate has been negative (Dubreuil *et al.*, 1990a); however, a carbohydrate biotinylation assay stained p57 from strain JD24 but not MT239 (Senson and Stevenson, 1999). Since p57 does not reassemble on to strain MT239, a potential role for carbohydrate in the reassociation of p57 to the bacterial cell surface has been proposed (Senson and Stevenson, 1999). However, the nature of this association has not been defined biochemically. Based on the gene sequence analysis, the theoretical isoelectric point (pI) of p57 is 4.6. The amino acid composition of the protein is rich in glycine, valine, tryptophan, alanine and serine (Chien *et al.*, 1992). Of the amino acids, 38% are hydrophobic, possibly contributing to the hydrophobic nature of *R. salmoninarum* cells. Fimbriae are often hydrophobic (Jones and Isaacson, 1984) and it has been suggested that the short peritrichous fimbriae observed using electron microscopy might be composed of p57 (Dubreuil *et al.*, 1990b). A fimbriae structure is not required for biological activity, as the p57 monomer is sufficient for agglutinating activity (Wiens *et al.*, 1999).

A number of investigators have shown that p57 is unstable and susceptible to degradation while attached to the bacterial cell surface, secreted into culture or secreted during infection (Dubrieul *et al.*, 1990a; Griffiths and Lynch, 1991; Rockey *et al.*, 1991b). Freezing or heating decreases the stability of the 57 kDa protein (Griffiths and Lynch, 1991). Larger amounts of lower molecular weight bands are found in aged culture supernatants. Addition of the serine protease inhibitor, phenylethylsulfonyl fluoride, inhibits proteolysis (Griffiths and Lynch, 1991; Rockey *et al.*, 1991b). In an attempt to identify the responsible protease, Griffiths and Lynch (1991) resolved the ECP using two-dimensional electrophoresis. Breakdown of the 57 kDa protein occurred after the first dimension resolution and was apparent in the 57 and 33–37 kDa bands, suggesting that p57 might be autolytic. In contrast, Rockey *et al.* (1991b) identified a high molecular weight protease independent of p57. This protease has low activity against p57 at 17°C and is highly active at 37°C. This protease is inhibited by phenylethylsulfonyl fluoride, methanol, ethanol and 10 min incubation at temperatures higher than 65°C. Purified p57 preparations do not exhibit degradation, suggesting that p57 is not autolytic or that the autolytic activity has been destroyed during purification (Rockey *et al.*, 1991b; Wiens *et al.*, 1999). Addition of the *R. salmoninarum* protease to purified p57 at 17°C yielded a spectrum of its breakdown products similar in molecular mass and antigenicity to those seen in the extracellular protein (Rockey *et al.*, 1991b). Incubation of p57 at 37°C results in complete degradation into numerous smaller molecular weight bands. This suggests that either the protease has high activity at elevated temperatures, or p57 is more susceptible to degradation due to denaturation and increased proteolytic site exposure. The physiological function of the protease is unknown, but one possibility is that it modulates the amount of functionally active p57 on the bacterial cell surface. Interestingly, p57 processing is reduced in iron-restricted cultures (Grayson *et al.*, 1995c). Since iron-limited conditions occur in fish serum, this may facilitate expression or stability of p57 early during the infectious process prior to intracellular invasion. To date, we have been unable to identify the responsible protease from *in silico* genome analyses.

The *in vivo* function of p57 is uncertain, but its concentration in fish tissues increases during disease progression (Turaga *et al.*, 1987b). The protein has been associated with a number of biological activities *in vitro*, which may be responsible for some of the observed pathology. Among these are mammalian erythrocyte agglutination (Daly and Stevenson, 1987, 1990), salmonid spermatocyte and leucocyte agglutination (Daly and Stevenson, 1989; Wiens and Kaattari, 1991), non-specific suppression of salmonid antibody responses (Rockey *et al.*, 1991b; Fredriksen *et al.*, 1997), suppression of phagocytic cell bactericidal activity (Siegel and Congleton, 1997) and suppression of respiratory burst activity (Densmore *et al.*, 1998). In an interesting study, Brown *et al.* (1996) injected p57 into coho salmon eggs, then fertilized, raised fry and then challenged. Fish injected with 100 ng p57 demonstrated significantly higher cumulative percent mortality than those from the saline-injected eggs. The p57-injected fish produced lower levels of antibodies against p57, although not against whole cells, and exhibited a lower phagocytic response. These data suggest that early egg exposure may result in long-term immunodepression and a decreased ability to resist subsequent challenge with *R. salmoninarum*.

Innate immunity

The analysis of fish immune responsiveness to *R. salmoninarum* is an active area of research and provides a natural system for understanding lower vertebrate immune mechanisms against a facultative intracellular pathogen. Study of pedigree fish populations and analysis of survival following challenge are a powerful combination for study of innate immune mechanisms. From

post-challenge survival measurements and pedigree relationships, the amount of environmental variation versus genetic influence can be determined as a heritability (h^2) estimate. Heritability is reported on a scale from 0 (completely environmental or non-genetic) to 1 (complete genetic influence). Studies measuring genetic variation in resistance of Atlantic and Pacific salmon to BKD indicate a wide variation in heritability. In coho salmon, the estimated sire component of survival heritability was 0.90±0.26 and the time to death was 0.26±0.10 (Withler and Evelyn, 1990). In Chinook salmon, low to moderate heritability estimates were obtained from three populations in British Columbia (Beacham and Evelyn, 1992), while high heritability estimates for survival (h^2=0.89±0.26) and days to death (h^2=0.35±0.16) were reported for a Columbia River stock of Chinook salmon (Hard *et al.*, 2006). Purcell *et al.* (2008) indicated that Lake Michigan stock Chinook salmon (from Wisconsin) were much more resistant than a closely related progenitor stock from the Green River. The Lake Michigan stock underwent one of the largest known natural die-offs of BKD during the spring of 1988 to 1992 and Purcell *et al.* (2008) proposed that the differing resistance between the two populations was strong evidence for pathogen-driven selection acting on a natural population. The generally high heritability of resistance that varies between species and populations highlights the potential for BKD to influence the genetic structure of a natural population.

The mechanisms of BKD resistance are unclear. The transferrin genotype correlates with disease resistance in coho salmon (Suzumoto *et al.*, 1977). Fish of AA genotype are the most susceptible, of AC genotype are intermediate and of CC genotype are the most resistant to disease challenge. Addition of exogenous iron did not increase mortality and thus these data taken together suggested that the transferrin genotype was an indirect marker for resistance. In a small study of 20 spawning Atlantic salmon, homozygosity at the major histocompatibility IIB locus correlated with higher bacterial antigen loads and increased susceptibility (Turner *et al.*, 2007).

Another approach to identify immune mechanisms is to measure changes in host and pathogen gene expression following infection. Using qualitative PCR assays, Grayson *et al.* (2002) identified changes in macrophage gene expression and bacterial gene expression in purified kidney cultures infected *in vitro*. The macrophage gene expression profile indicated rapid activation of the inflammatory response including upregulation of interleukin-1β (*IL-1β*), major histocompatibility complex class II (*MHC II*), inducible cyclo-oxygenase (*Cox-2*), inducible nitric oxide synthase (*iNOS*), CXC chemokine receptor 4 (*CXC-R4*), CC chemokine receptor 7 (*CC-R7*) and *Mx 1–3* genes. Tumour necrosis factor-α (*TNF-α*) expression initially decreased at 2 h and then recovered by 24 h post-infection. The bacterium constitutively expressed *msa* gene message, while haemolysins *hly* and *rsh* gene message decreased. Interestingly, in a second series of experiments, injection of *msa2*-expressing plasmid construct into trout muscle resulted in immune suppression of *IL-1β*, *Cox-2* and *MHC-II* message and increased abundance of *TNF-α* message. The *msa* DNA vaccine construct provided no protection and exacerbated mortality following challenge. The investigators propose a model in which *R. salmoninarum* activates an inflammatory response but survives initial contact with macrophages by avoiding and/or interfering with *TNF-α*-dependent killing pathways. Excess *TNFα* production results in granuloma formation.

Using an elegant study design, Rhodes *et al.* (2009) examined gene expression changes in Chinook salmon kidney tissue following infection with an attenuated strain (MT239) or a virulent strain (ATCC 33209). Suppression subtractive hybridization identified 132 expressed sequenced tags enriched in tissue from MT239 infected fish that had high sequence similarity to immune response genes. Of the ESTs identified, 20 were selected for expression analysis at 24 and 72 h after challenge and 17 were differentially regulated. Interestingly, four genes (*INF-inducible GBP*, *VLIG1*, *GAS6* and *VRK2*) were upregulated in fish exposed to MT239, but downregulated or

unchanged in fish exposed to 33209. Four genes (*caspase 8*, *IKBa*, *p47^{phoX}* and *EMR/CD97*) were upregulated more rapidly in fish exposed to MT239 as compared with fish exposed to ATCC33209. Both sets of genes have potential to be useful biomarkers of favourable immune responsiveness of infected stocks, or as surrogate markers for measuring vaccine efficacy.

A large number of putative upregulated proteins in both the liver (n=95) and kidney (n=46) were identified using proteomic analysis of Atlantic salmon infected for 25 days with an undescribed strain of *R. salmoninarum* (Booy *et al.*, 2005). A smaller number of downregulated proteins were identified in the liver (n=9) and kidney (n=43). Of note, immune proteins IL-8, β2-microglobulin, NADPH oxidase and MxA were increased. The apparent absence of common regulated genes/proteins highlights the complexity of working with multiple species and incomplete immune-response pathways in fish (Grayson *et al.*, 2002; Booy *et al.*, 2005; Rhodes *et al.*, 2009). The future development of comprehensive host- and pathogen-specific microarrays is critical to understanding fully the global transcriptional and proteomic changes that occur during infection.

Adaptive immunity: humoral and cell-mediated

Circumstantial evidence suggests that some infected salmonids mount a protective immune response. Munro and Bruno (1988) described a natural epizootic of *R. salmoninarum* in Atlantic salmon during smoltification which resulted in 18% cumulative mortality. Subsequently, fish were Gram- and IFAT-negative up to 69 weeks post-seawater transfer, and 100% of the fish had agglutinin response and demonstrated a resolution of granulomatous lesions. These observations clearly indicate exposure and resolution of the infection. Antibody responses are induced by intraperitoneal injection of killed *R. salmoninarum* (Evelyn, 1971) or by immersion challenge (Jansson and Ljungberg, 1998; Jansson *et al.*, 2003). However, antibody responses are generally slow and dependent on the fish-rearing temperature (Alcorn *et al.*, 2002). Fish produce a robust antibody response against p57 as well as to peptidoglycan, but not to the galactose-rich polysaccharide (Sorum *et al.*, 1998a). *R. salmoninarum* is resistant to normal and immune serum killing (Bandin *et al.*, 1995) and, at present, there is no evidence that humoral immunity is protective. In fact, antibody production may be counterproductive. Chronically infected trout and Chinook salmon both display thickened glomerular basement membranes, electron-dense subendothelial deposits and deposition of fish immunoglobulin (Young and Chapman, 1978; Sami *et al.*, 1992; Lumsden *et al.*, 2008). These pathological signs may represent a type III hypersensitivity reaction, which is the deposition of antibody–antigen complex followed by complement mediated host tissue destruction. However, there are no reports of the isolation of antibody–antigen complexes or measurement of complement deposition. Attempts to induce experimental type III hypersensitivity by immunization of rainbow trout with formalin-killed *R. salmoninarum* ATCC 33209 failed to reproduce the glomerulopathy observed in chronically infected animals (Lumsden *et al.*, 2008).

Renibacterium has emerged as a relevant system for understanding mechanisms of fish cellular response to infections (Ellis, 1999; Campos-Perez *et al.*, 2000; Grayson *et al.*, 2002; Jansson *et al.*, 2003). A pathogen-induced cell population is present in rainbow trout spleens and has been identified using the 1C2 MAb (Jansson *et al.*, 2003). This MAb recognizes the TCR β-chain and a subpopulation of Ig$^+$ cells. 1C2$^+$ cells initially increase after challenge, then decrease by 10 weeks post-challenge, but can be re-stimulated after a secondary challenge with heat-killed bacteria. Inducible nitric oxide synthase mRNA is upregulated robustly after *Renibacterium* injection or bath challenge (Campos-Perez *et al.*, 2000) and it is likely that production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI) is a

critical killing mechanism utilized by the adaptive immune response. Determination of the precise nature and function of response cells and killing mechanisms will be an important advance in understanding cell-mediated immunity in fish.

Control, Treatment and Epizootiology

Husbandry methods and good practice

In spite of the high prevalence of BKD, techniques for its control are limited, in both number and efficacy (Elliott *et al.*, 1989; Warren, 1991). Management strategies designed to control BKD have included reducing stress to fish, quarantining infected stocks, dietary modification, chemotherapy, culling of infected broodstock and/or total hatchery depopulation and sterilization (reviewed in Elliott *et al.*, 1989).

Chemotherapy

Chemotherapeutic control of BKD is widely recognized as a difficult problem (Elliott *et al.*, 1989). Erythromycin is thought to be efficacious when injected into pre-spawning adult broodfish or incorporated into the feed of juvenile fish. Erythromycin phosphate, when injected (11 mg/kg) at 21- to 30-day intervals until spawning, reduces adult Chinook salmon mortality (Groman and Klontz, 1983). Care should be exercised as higher doses of 20–40 mg/kg can cause antibiotic-induced jaundice and fish mortality (Moffitt and Kiryu, 1999; Haukenes and Moffitt, 2002). Antibiotic injection is effective in reducing levels of *R. salmoninarum* within the egg (Evelyn *et al.*, 1986b; Brown *et al.*, 1990) and feeding is an effective method for delivering erythromycin to juvenile fish. Erythromycin-medicated feed reduced the mortality of infected eastern brook trout (Wolf and Dunbar, 1959), rainbow trout (Austin, 1985) and Chinook salmon challenged experimentally with *R. salmoninarum* (Moffitt and Bjornn, 1989). A dose of 100 mg/kg body weight of erythromycin thiocyanate medicated fed for 28 days was the most effective treatment against a severe intraperitoneal challenge (Peters and Moffitt, 1996). The use of erythromycin in the USA is limited and not approved as a therapeutic drug for fish culture. It is only available as an investigational new animal drug (INAD) through the Food and Drug Administration (Moffitt, 1992). In some instances, no cost benefit has been found to using erythromycin in place of oxytetracycline for BKD treatment of net-pen cultured fish (Kent, 1992). If erythromycin is approved, chemotherapy is still problematic as it is not completely effective in curing infected fish or preventing vertical transmission (Austin, 1985; Brown *et al.*, 1990). Additionally, the demonstration of bacterial resistance to erythromycin *in vitro* (Bell *et al.*, 1988), the strictly bacteriostatic mechanism of action and the prophylactic treatment of juvenile fish and adult broodstock increase the probability that the emergence of resistant strains may be a future concern. Recently, *R. salmoninarum* with reduced susceptibility to macrolide antibiotics (erythromycin and azithromycin) were isolated from Chinook salmon recovering from multiple antibiotic treatments as part of an endangered stock restoration programme (Rhodes *et al.*, 2008). The mechanism of decreased susceptibility is unknown but is not due to mutation in the 23S rRNA or in the *L4* or *L22* genes.

Broodstock segregation

The lack of a completely efficacious vaccine (Munro and Bruno, 1988; Kaattari and Piganelli, 1997) and potential problems with chemotherapy have stimulated research to develop novel methods for the control of BKD. Currently, the most widespread approach is to cull gametes from infected adults (Armstrong *et al.*, 1989; Elliott *et al.*, 1989). The premise of this control method is that stopping vertical transmission reduces mortality and increases fish survival and return. In a seminal study, effects of adult segregation were measured using spring Chinook salmon returning to Dworshak National Fish Hatchery in Idaho, USA (Pascho *et al.*, 1991b). Spawners with high or low levels of *R. salmoninarum*, as determined using

ELISA and FAT, were segregated into two groups and gametes and offspring reared separately. Offspring from highly infected adults had a higher cumulative pond mortality (17% versus 5%) and higher prevalence of infection at the time of release (85% versus 62%) than offspring from the adults with low antigen levels. These results demonstrated that broodstock segregation could be a useful strategy to control BKD even in situations where untreated hatchery water was used. However, the significant prevalence of infection in the BKD low progeny also suggests that horizontal transmission is important under hatchery conditions or that fish with even marginal levels of *R. salmoninarum* infection are capable of promulgating transmission. Segregation has also shown promise for increasing juvenile survival during downriver migration and entry into seawater (Pascho *et al.*, 1993; Elliott *et al.*, 1995). An encouraging observation was made at an Oregon hatchery that reported that spring Chinook offspring from low BKD parents returned at twice the rate as compared with fish from parents with medium to high BKD (Warren, 1991). Recently, there have been two reports of the long-term implementation of culling combined with antibiotic prophylaxis (Gudmundsdóttir *et al.*, 2000; Meyers *et al.*, 2003). After several years, the prevalence of infected *R. salmoninarum* infected fish dropped from 35% to less than 2% at two sea ranches in Iceland (Gudmundsdóttir *et al.*, 2000). In Alaska, where three Chinook and two coho salmon broodstock populations were monitored, most of the stocks showed reductions in *R. salmoninarum* prevalence and no major epizootics were recorded (Meyers *et al.*, 2003). Both reports recommend culling as a management strategy; however, the precise improvement that may be expected is unknown, as control populations of fish are not kept at the same sites. The incidence of BKD varies significantly from year to year and the factors that cause fluctuation are not understood (VanderKooi and Maule, 1999; Meyers *et al.*, 2003). The maturation of adult Chinook salmon in seawater may also reduce the prevalence of *R. salmoninarum* (Meyers *et al.*, 1999). In summary, culling is a strategy for the control of BKD but not for eliminating *R. salmoninarum* from an affected population. Removal of feral fish that contaminate the water supply is probably an important consideration if complete elimination is the objective (Meyers *et al.*, 2003). Importantly, genetic analysis suggests that selective culling of female broodstock with high antigen titres does not affect resistance of progeny to challenge with BKD or vibriosis (Hard *et al.*, 2006).

Vaccination

BKD has been a difficult disease to prevent by vaccination. Experimental progress has been slow due to the chronic nature of the disease and the long period required for vaccine trials. In addition, many of the most important fish stocks relevant for vaccine trials contain low levels of infection, thus complicating the interpretation of results. Conventional approaches to vaccine development, which have been highly successful against Gram-negative bacteria, have been generally disappointing when applied to *R. salmoninarum*. The protection provided by vaccination with extracellular products or formalin-killed bacterial cells is limited in efficacy (Munro and Bruno, 1988; Sakai *et al.*, 1989b; Evenden *et al.*, 1993; Kaattari and Piganelli, 1997). There are several novel approaches to vaccination that have been tried. One approach removed bacterial-associated p57 implicated in immune modulation and pathological responsiveness (Kaattari and Piganelli, 1997). During an attempt to inactivate ECP using heat treatment, a fortuitous observation identified that incubation of viable *R. salmoninarum* at 37°C stimulated that activity of an endogenous protease, thereby removing p57 from the cell surface (Piganelli *et al.*, 1999a). Vaccination with whole cells, delivered to coho salmon either by injection or in the feed, resulted in delayed mortality and reduced infection (Piganelli *et al.*, 1999b). A different approach was taken by Daly *et al.* (2001), who identified spontaneous *R. salmoninarum* mutants that no longer required cysteine supplementation for growth. Intraperitoneal challenge demonstrated mutant

attenuation and vaccination provided significant protection against subsequent challenge. The nature of the spontaneous mutation(s) and the possible reversion to a virulent form is unclear. Griffiths *et al.* (1998) reported that vaccination with *Arthrobacter*, a closely related microorganism, was capable of stimulating cross-protective immunity. A strain was isolated from Chinook salmon kidney and classified as *Arthrobacter* spp., based on rRNA sequence and analytical profile index (API). The strain grew on tryptic soy agar plates (TSA), a characteristic unusual for *R. salmoninarum*, and lacked p57, as determined using antibody staining. This strain produced a carbohydrate that cross-reacted with *Renibacterium*, possibly explaining its efficacy. This strain is an approved, commercial vaccine, named Renogen (Griffiths and Kira, 2003). A large-scale field trial with Atlantic salmon demonstrated reduced BKD mortality and increased growth (Salonius *et al.*, 2005). However, in experimental studies with Chinook salmon, Renogen provided only limited protection to fish challenged by intraperitoneal injection (Rhodes *et al.*, 2004b) or no protection to fish challenged by cohabitation (Alcorn *et al.*, 2005). Interestingly, in fish naturally infected with *R. salmoninarum*, a combination of Renogen and the attenuated *R. salmoninarum* strain MT239 increased survival significantly better than either preparation alone (Rhodes *et al.*, 2004b). This study is the first demonstration of a potential therapeutic effect of a whole cell vaccine and this strategy may be particularly relevant when it is necessary to vaccinate infected fish stocks. A review of vaccine use in Chilean aquaculture production from 1999 to 2003 indicated use of Renogen since 2001 but vaccine coverage was low, with only 5% of coho salmon vaccinated (Bravo and Midtlyng, 2007). In Chile, BKD often co-occurs with piscirickettsiosis and control of BKD is correlated with reduced *Piscirickettsia salmonis* (Salonius *et al.*, 2005; Bravo and Midtlyng, 2007). In summary, much work remains in developing a robust BKD vaccine that is reproducibly efficacious in multiple salmonid species. The major obstacles remain the limited understanding of protective immunity, the response against heterologous *R. salmoninarum* stains and the optimal route for vaccination. The development of DNA vaccines and the incorporation of immunostimulants may offer additional approaches to vaccination (Saki *et al.*, 1995; Grayson *et al.*, 2002).

Conclusions and Recommendations for Future Studies

Bacterial kidney disease continues to be an intractable disease of salmonid fish. The disease impacts aquaculture negatively in both the northern and southern hemispheres, and fully efficacious prophylactics and therapeutics that work in multiple salmonid species are not yet commercially available. Many basic questions remain and productive areas for future study include genomics, pathogenesis, transmission and host response. It is anticipated that the *R. salmoninarum* genome sequence will be a valuable tool for research scientists working to identify diagnostic, immunogenic and therapeutic targets. However, it should be emphasized that the sequence represents a 'snapshot' of the genetic information contained in a single bacterial clone isolated in 1984 from a Chinook salmon returning to the Willamette River in Oregon, USA. Additional isolate sequencing obtained from diverse locations and time points is warranted to understand the core genome fully, as well as genomic variation between strains. Genome sequencing is decreasing rapidly in cost, and software for processing this information is readily available. The genomic data have posed a number of intriguing questions. First, abundant pseudogenes and inaccurate start-site prediction underscore the need for global transcriptional studies and metabolic reconstruction. The recent development of direct transcript sequencing offers the opportunity to identify gene start sites and transcript abundance of *R. salmoninarum* grown under various culture conditions and during fish infection. *R. salmoninarum* contains genes

that appear to be horizontally acquired virulence factors, including capsular synthesis genes, haem acquisition operons, genes encoding possible haemolysins and the *msa* genes. Most of these genes do not have homologues in the closely related *Arthrobacter* spp. and, thus, they may form the basis of the niche differences between these bacteria. Further expression and biochemical analyses of these gene products will likely elucidate the molecular mechanisms of pathogenesis. It is also likely that a number of virulence determinants are not recognized in the genome sequence. The 992 ORFs absent in *Arthrobacter* spp. represent an initial list of candidates that should be targeted for further study. The putative sortase cleaved proteins are also a potential source of novel virulence factors for further study. Research on BKD pathogenesis will be facilitated by development of better genetic systems for rapidly creating isogenic mutants. Metabolic reconstruction may identify nutrients that may speed *R. salmoninarum* growth *in vitro*. In summary, the genome sequence increases our understanding of the pathogenesis of *R. salmoninarum* infection, but most of the host–microbe interactions in this system remain poorly understood.

Understanding the genetic regulation of the *msa* genes and other antigens will facilitate the design of optimal immunodiagnostic assays to identify infected or recovering fish. Specific and sensitive ELISAs have been developed for detecting antigen in juvenile and adult fish; however, the ELISA cannot detect *R. salmoninarum* antigen inside eggs (Kaattari *et al.*, 1993). New real-time PCR assays are useful for identifying minute amounts of *R. salmoninarum* DNA in tissues, ovarian fluid and eggs. Technical limitations, such as the sample preparation and mass screening techniques using PCR, still need to be overcome before such assays will be useful for widespread practical application. Sensitive and specific assays for the detection of *R. salmoninarum* are critical, as segregation of broodstock is an effective strategy for reducing mortality among offspring and increasing adult returns. Control of the disease is of utmost practical importance and, at present, culling infected gametes is the best option for reducing vertical transmission. However, this strategy is limited if horizontal transmission is possible via feral fish and the water supply is not biosecure.

Thus far, induction of immunity to BKD by vaccination has not been consistent. Vaccination experiments are technically difficult to design due to slow growth of the pathogen and the long challenge times required to observed mortality as an experimental end point. Horizontal transmission experiments as described by Alcorn *et al.* (2005) require 9 months to complete and thus this time commitment reduces significantly the numbers and combinations tested. Another complication when working with Chinook salmon is that it is difficult to obtain stock free of *R. salmoninarum*. If infected stock is used in experiments, it is unclear how these results compare with results from non-infected fish. Measuring antigen levels in tissues after immersion challenge with *R. salmoninarum* is an effective method for evaluating disease progression, and the identification of immunological tools for the early assessment of protective immunity is a promising area of study. Rhodes *et al.* (2009) have identified a suite of new genes in Chinook salmon that may be useful biomarkers. More work is needed to determine the role of these genes and whether they are also useful in other fish species. Comparative genomics of *Arthrobacter* and *Renibacterium* offer the opportunity to identify cross-reactive antigens. A total of 1562 ORF clusters were similar between *R. salmoninarum* and *Arthrobacter* spp. and this high level of genomic similarity may explain the efficacy of the commercial vaccine, Renogen, which is composed of a live, lyophilized *Arthrobacter*. Finally, there are only a limited number of approaches available for measuring specific cellular immunity. Overcoming this deficiency is likely to be critical for the assessment of salmonid immunity to BKD, since protection against other intracellular pathogens is mediated through the cellular component of the immune response.

Acknowledgements

This work was supported by Agricultural Research Service CRIS Project 1930-32000-002 (Host–Pathogen and Environmental Interactions in Cool and Cold Water Aquaculture). Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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10 *Enterococcus seriolicida* and *Streptococcus* spp. (*S. iniae*, *S. agalactiae* and *S. dysgalactiae*)

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The increasing demand for fish production has resulted in intensive culture of many species of fish. Outbreaks of bacterial, viral and/or parasitic diseases are more common when fish are reared at high densities. Among Gram-positive bacteria, streptococcal infections, which increased during the last decade as a consequence of the intensification of aquaculture, are responsible for significant economic losses in the fish farm industry. In particular, *Enterococcus seriolicida* and *Streptococcus iniae* are very important pathogens in cultured marine and freshwater fish. Both pathogens show similar clinical disease: *E. seriolicida* is the most damaging bacterial pathogen in cultured marine fish in Japan, particularly in yellowtail (*Seriola quinqueradiata*) and, recently, in rainbow trout (*Oncorhynchus mykiss*) reared in Europe. *S. iniae* causes recurrent outbreaks of disease in many species of seawater and freshwater cultured fish around the world.

Enterococcus seriolicida/ *Lactococcus garvieae*

Introduction

An epizootic *E. seriolicida* in 1974 caused heavy losses in yellowtail cultured in Shikoku Island, Japan (Kusuda *et al.*, 1976). The disease subsequently spread all over the country. For many years, the causative agent was described as belonging to the genus *Streptococcus* (Kusuda and Kawai, 1982), but later it was included in the genus *Enterococcus* (Kusuda *et al.*, 1991). However, more recent studies have demonstrated a close relationship between *E. seriolicida* and *Lactococcus garvieae* (Domenech *et al.*, 1993; Eldar *et al.*, 1996). Enterococcosis/lactococcosis is the most important bacterial disease in cultured yellowtail in Japan and it causes severe problems in trout farms in France, Greece, Israel, Italy, Portugal, Spain and Turkey.

The disease agent

The disease caused by *E. seriolicida*/*L. garvieae* is now called lactococcosis, or enterococcal infection or enterococcosis, instead of streptococcal infection or streptococcosis.

Recently, it has been demonstrated that *E. seriolicida* is a junior synonym of *L. garvieae* (Domenech *et al.*, 1993; Teixeira *et al.*, 1996). However, the classification of nonmotile Gram-positive cocci, such as *Enterococcus*, *Lactococcus* or *Streptococcus*, is still being discussed. *E. seriolicida* (*seriolicida* means yellowtail killer)/*L. garvieae* is a 0.7×1.4 µm Gram-positive ovoid-shaped

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bacterium which forms short chains (Fig. 10.1). It is a facultative anaerobic, non-motile, non-spore-forming coccus.

The bacterium grows on infusion (brain–heart infusion, BHI) nutrient agar, 40% bile agar and on 0.1% methylene-blue milk, but does not grow on MacConkey agar, *Salmonella–Shigella* agar or on *S. faecalis* broth (Kusuda *et al.*, 1991). The colonies are small, white, round and regular.

Growth occurs between 10 and 45°C, 0 and 6.5% sodium chloride (NaCl) and pH 4.5 and 9.6. Optimal conditions are at 37°C, 0% NaCl and pH 7.6. Catalase, cytochrome oxidase, casein digestion and potassium tellurite reduction are negative. Moreover, there is no production of indole, hydrosulfide, ammonium or gelatinase, whereas the bacterium reduces methylene blue and nitrate. The methyl red test, Voges–Proskauer reaction, 2,3,5-triphenyltetrazolium chloride reduction and esculin hydrolysis are positive. *E. seriolicida/L. garvieae* is fermentative, producing acid from D-glucose, D-mannose, galactose, D-fructose, trehalose, maltose, cellobiose, dextrin, sorbitol, mannitol, salicin and esculin, but not from D-xylose, D-arabinose, L-rhamnose, sucrose, lactose, melibiose, raffinose, melezitose, starch, glycogen, glycerol, inositol or adonitol. Alpha-haemolysis is detected in blood agar (Kusuda *et al.*, 1991).

Serological tests have been performed by Kusuda *et al.* (1982). Non-agglutinating (capsulated, KG– phenotype) and agglutinating (non-capsulated, KG+ phenotype) strains have been recognized (Alim *et al.*, 1996; Okada *et al.*, 2000). The antigen extracted by the hot-acid method of Lancefield (1933) is different from the antigen of groups A, B, C, D, E, F, G, H, K, L, M, N, O and MG of Lancefield's classification.

Geographical distribution and host range

The bacterium survives throughout the year and may be found in seawater containing high levels of organic matter (Kusuda, 1990). In intensively cultured fish, an environmental stress predisposes them to the disease; particularly water temperature is considered the most important predisposing factor for the onset of the disease during the hottest period of the year (Kusuda, 1990).

E. seriolicida/L. garvieae is the aetiological agent of disease in both marine and freshwater fish. It was first isolated from

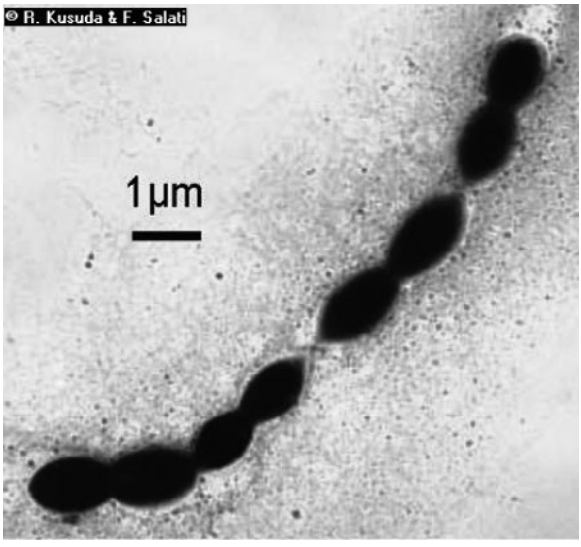


Fig. 10.1. Transmission electron micrograph of *Enterococcus seriolicida/Lactococcus garvieae* cultured on brain–heart agar at 37°C for 24 h.

diseased cultured yellowtail outbreak that gave the name to the bacterium and also from an epizootic in cultured eel (*Anguilla japonica*) (Kusuda *et al.*, 1978). Furthermore, the bacterium was detected in seawater, in mud and in the intestine of wild fish, such as Spanish mackerel (*Scomber japonicus*), black scraper (*Novodon modestus*), striped mullet (*Mugil cephalus*), Japanese horse mackerel (*Trachurus japonicus*) and Red Sea wrasse (*Coris aygula*), which could be considered as healthy hosts facilitating transmission of the disease (Kusuda and Kawai, 1982; Colorni *et al.*, 2003). A disease caused by an *Enterococcus*-like bacterium was also reported in ayu (*Plecoglossus altivelis*), tilapia (*Tilapia nilotica*) and in cultured turbot (*Scophthalmus maximus*), flounder (*Paralichthys olivaceus*), gilthead sea bream (*Sparus aurata*) and, recently, in farmed Adriatic sturgeon (*Acipenser naccarii*) (Kusuda *et al.*, 1978; Ceschia *et al.*, 1992; Kusuda and Salati, 1993; Kim and Lee, 1994; Toranzo *et al.*, 1994; Salati *et al.*, 1996). Moreover, since the summer of 1991, several outbreaks of disease have been reported in rainbow trout reared in Italy, Spain and France, then in Portugal, Greece and Iran (Ceschia *et al.*, 1992; Ghittino and Prearo, 1992; Palacios *et al.*, 1993; Mùzquiz *et al.*, 1999; Pereira *et al.*, 2004; Savvidis *et al.*, 2007; Soltani *et al.*, 2008) and also in Australia and South Africa (Carson et *al.*, 1993), demonstrating that the geographical distribution of this bacterium and the disease outbreaks are strongly related with high water temperatures. Furthermore, the bacterium can cause disease in ponds of cultured giant freshwater prawn (*Macrobrachium rosembergii*) (Cheng and Chen, 1998) and has also been isolated from a diseased wild bottlenose dolphin (*Tursiops truncatus*) from Kuwait Bay (Evans *et al.*, 2006), demonstrating that it can trespass the species barrier easily, passing from teleosts to crustaceans to mammals.

Economic importance of the disease

In 1974, an epizootic *E. seriolicida* caused heavy losses in yellowtail cultured in Shikoku Island, Japan (Kusuda *et al.*, 1976). Infection has subsequently spread across the country and has become the most important disease in cultured yellowtail. Enterococcosis/lactococcosis is the most important bacterial disease in cultured yellowtail in Japan: in 1992, losses were *c.*5954 t out of a total production of *c.*130,876 t (Department of Research, Japanese Ministry of Fisheries, 1994). In 2007, in spite of vaccines being used since 2005, enterococcosis/lactococcosis was still one of the most important bacterial diseases in cultured yellowtail in Japan, showing losses around 10,000 t (Prof. Y. Takahashi, Shimonoseki University of Fisheries, Shimonoseki, Japan, personal communication, 2008).

In the past decade, enterococcosis/lactococcosis has also become the major bacterial fish disease in trout farms in Austria, France, Greece, Israel, Italy, Portugal, Spain and Turkey, where it causes severe problems. Italian trout production has, for instance, declined from 51,000 t in 1997 to 38,000 t in 2003, primarily because of this disease; in particular, in the farms of north-east Italy, mean mortality ranged from 30 to 60% of fish at commercial size (Fabris, 2004). Moreover, the same high mortality was reported by Soltani *et al.* (2008) during the summer of 2006 in commercial-size trout cultured in south-west Iran.

Diagnostic methods

Enterococcosis/lactococcosis is an infectious systemic disease characterized by haemorrhagic septicaemia. Diseased fish show the following clinical signs: anorexia, lethargy, loss of orientation and bilateral exophthalmia (Figs 10.2 and 10.3). Other signs of the disease are petechiae on the inside wall of the opercula and congestion of blood vessels in the pectoral and caudal fins (Fig. 10.5).

The abdomen can be distended and the peritoneal cavity often contains bloody ascitic liquid. The gross appearance includes congestion and haemorrhage of the brain, kidney, intestine, liver and spleen, and

sometimes a haemorrhagic enteritis (Figs 10.4 and 10.5). Little is known about the haematological variation in diseased fish, although Kobayashi *et al.* (2004) have shown that in cultured rockfish (*Sebastes schlegeli*) the haematocrit values are significantly lower than those of healthy fish.

The histopathological features of enterococcal infection show evidence of pathogen proliferation and degeneration and necrosis



Fig. 10.2. Infection in yellowtail (*Seriola quinqueradiata*) showing bilateral exophthalmia.



Fig. 10.3. Infection in red sea bream (*Pagrus major*) showing bilateral exophthalmia.

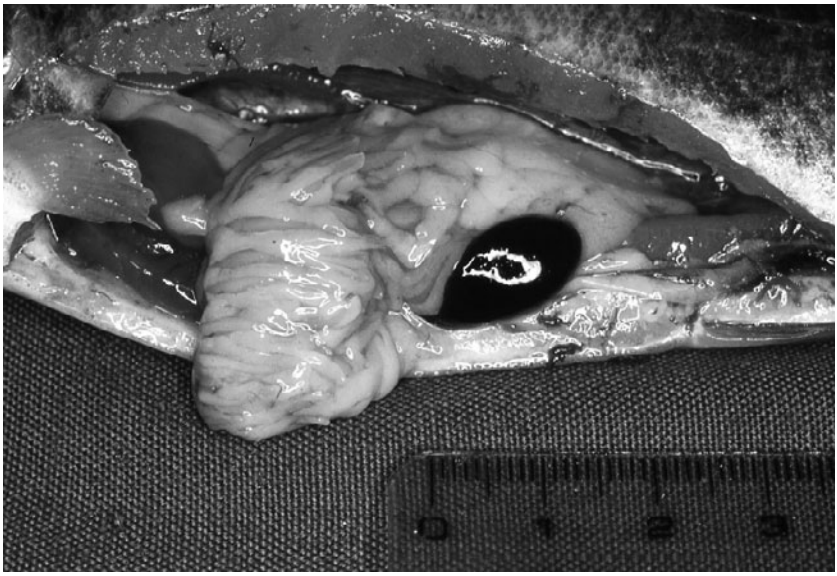


Fig. 10.4. Infection in rainbow trout (*Oncorhynchus mykiss*) showing hypertrophy of the spleen, hyperaemia of the intestine and haemorrhage in the muscular tissue (photograph courtesy Drs L. Alborali and C. Salogni).



Fig. 10.5. Eels (*Anguilla japonica*) showing lesions caused by *Enterococcus seriolicida* / *Lactococcus garvieae*.

in many organs, such as eyes, gills, heart, spleen and skin (Egusa, 1978; Kobayashi *et al.*, 2004).

An accurate identification is required to carry out a proper epidemiological survey, because fish with warmwater 'streptococcosis' exhibit similar lesions and clinical signs. The definitive diagnosis of the aetiological agent is based on the microbiological identification of the pathogen. The isolation of the bacterium and the characteristics of the colonies contribute to the diagnosis. To confirm the identification of the aetiological agent, it is necessary to perform culture-based tests, such as tolerance to bile esculin and tellurite and growth in 6.5% NaCl broth, and biochemical tests, such as specific carbohydrate utilization and pigment production. Nevertheless, biochemical identification of the bacterium can be difficult when commercial kits such as API 20 Strep, rapid ID 32 Strep system or API 50 galleries are used, due to the lack of specific documentation and well-defined numerical profiles.

The identification of the aetiological agent can also be performed by fluorescent antibody (FA) techniques (direct and/or indirect methods) (Kusuda and Kawahara, 1987; Kawahara *et al.*, 1989a). Studies on the comparison between detection and diagnosis using standard culture and FA techniques demonstrate that the latter are more reliable and faster. They also show mixed infection, very difficult to detect with culture methods (Kawahara *et al.*, 1986). A sandwich enzyme-linked immunosorbent assay (ELISA) has been developed to quantify the humoral response in yellowtail (Matsubara *et al.*, 1985), but agglutinating antibody titres are used more often for this purpose.

Biomolecular diagnosis, restriction fragment length polymorphism (RFLP) and, during the past decade, PCR assays on 16S rRNA and amplified rRNA gene restriction analysis (ARDRA) have been developed for the identification of *E. seriolicida*/*L. garvieae* (Zlotkin *et al.*, 1998a; Aoki *et al.*, 2000; Mata *et al.*, 2004; Michel *et al.*, 2007). However, other studies have shown that PCR on *sodA* gene constitutes a more discriminative target sequence than *16S* rRNA gene (Poyart *et al.*, 2000). Furthermore, a number of techniques for recognizing *E. seriolicida*/*L. garvieae* have been published, such as the ID method based on reverse chequerboard hybridization to chaperonin (*Cpn60*) gene sequences, suggested by Goh *et al.* (2000), and the PCR amplifications of the 16S–23S rDNA intergenic spacer regions (ISR) using primers from highly conserved flanking regions within the *16S* and *23S* rRNA gene proposed by Hassan *et al.* (2003a).

Genome structure and transcription

Guanine-plus-cytosine content of deoxyribonucleic acid (DNA) is 44 mol % (Kusuda *et al.*, 1991). *E. seriolicida*/*L. garvieae* plasmid pKL0018 complete sequence may be found on GenBank accession number AB290882. Genetic characterization of *E. seriolicida*/*L. garvieae* using random amplified polymorphic DNA (RAPD) technique established that there were three genetic groups: Spanish, Portuguese, English and Turkish (group A), Italian and French (group B) and Japanese (group C) (Ravelo *et al.*, 2003).

Few studies have been performed to investigate the genetic relatedness between *E. seriolicida*/*L. garvieae* strains from fish and other animals, dairy samples and from terrestrial plants. The results showed that only isolates from *S. quinqueradiata* had strong pathogenicity to yellowtail and were susceptible to bacteriophages, while isolates from rainbow trout showed weak pathogenicity to yellowtail (Kawanishi *et al.*, 2006). The isolates from terrestrial animals and from dairy samples were generally not related to those collected from fish (Kawanishi *et al.*, 2006; Foschino *et al.*, 2008), and plant isolates were non-susceptible to bacteriophages and were non-capsulated and non-pathogenic towards yellowtail and mice (Kawanishi *et al.*, 2007).

Drug resistance mechanisms of 146 strains isolated in Japan from 1999 to 2006 have been studied by Maki *et al.* (2008): the study revealed that transferable plasmids encoding for resistance to

erythromycin, lincomycin and tetracycline were widely distributed and were conserved regardless of the area and/or season of isolation.

Pathogenesis and immunity

The most important predisposing factors for disease development in intensively cultured fish are environmental stress, particularly high water temperatures and high level of organic matter (Kusuda, 1990). The disease is transmitted horizontally (Kusuda, 1990). *E. seriolicida*/*L. garvieae* may be found in large number in the intestine of fish and, in stressful conditions, the bacterium spreads rapidly and colonizes the intestinal tract and kidney (Kusuda and Kimura, 1978; Kusuda and Kawai, 1982). Mortality varies with fish species and water temperature. In yellowtail, the mortality range is about 70–80% when the temperature is above 20–22°C. In trout, mortality varies between 50 and 60% when water temperature is higher than 17–18°C (Alborali and Carboni, 1997).

The literature describing the histopathology attributed to enterococcal infection is limited to that of Egusa (1978), who has found that in yellowtail there is proliferation of the pathogen and degeneration and necrosis of many tissues in the heart, gills, skin, spleen and eyes.

There are numerous reports on toxins produced by haemolytic streptococci in mammals, and the production of extracellular erythrogenic and pyrogenic toxins is well documented. However, there are only a few studies on toxins produced by cocci pathogenic to fish (Kusuda and Kimura, 1978; Kimura and Kusuda, 1979, 1982). Studies on the extracellular products of *E. seriolicida*/*L. garvieae* show that they are correlated to haemolytic activity and seem to induce specific clinical signs of the disease. Furthermore, the haemolytic activity reported for the extracellular products is similar to that of streptolysin O (SLO), because it is activated by cysteine. On the other hand, the intracellular products are correlated to leucocidal activity, which mainly affects phagocytic cells, and they seem to have cytotoxic effects. The lethal toxic factor(s) is probably of a proteinaceous nature, because it is inactivated by protease treatment (Kusuda and Hamaguchi, 1988, 1989). Other studies showed that the pathogenicity of *E. seriolicida*/*L. garvieae* appeared to be related to the agglutination pattern, because it was demonstrated using a slide agglutination test, and that non-agglutinating strains showed a higher pathogenicity than agglutinating ones (Alim *et al.*, 1996). It was also demonstrated that the cell surface might play an antiphagocytic role in *E. seriolicida*/*L. garvieae* infection (Yoshida *et al.*, 1996). However, the pathogenic mechanisms of this bacterium are poorly understood: few studies have been performed to identify its virulence factors (Menéndez *et al.*, 2007).

The immune response of fish to the bacterium and the production of specific antibodies in both serum and intestinal mucus of naturally infected yellowtail were investigated by Kusuda and Takagi (1983). Agglutination of cellular antigens and passive haemagglutination of endotoxins and exotoxins of *E. seriolicida*/*L. garvieae* were demonstrated; however, antibody production was low. The intestine is an important site for protection against enterococcal infections; this is demonstrated by the production of agglutinating antibody in the intestinal mucus. Studies on cell-mediated response in yellowtail injected with formalin-killed *E. seriolicida*/*L. garvieae* cells showed an increased and significantly enhanced phagocytic activity and phagocytic index of the head-kidney phagocytes. While the addition of the complement did not increase phagocytosis significantly, when specific antibodies were added together with the complement phagocytosis did increase significantly. These results indicate the activation of the complement classic pathway by the antigen–antibody reaction and the importance of opsonization and phagocytosis in the defence mechanisms in yellowtail (Kusuda and Tanaka, 1988). In rainbow trout, it has been reported that specific antibodies increased the bactericidal activity of

macrophages against virulent *E. seriolicida*/*L. garvieae* cells in the absence of complement (Barnes *et al.*, 2002). Phagocytosis of pronephros cells from peptidoglycan-fed yellowtail showed a higher phagocytic index than the controls 1 day after feeding, but no differences were recorded 5 days after feeding (Itami *et al.*, 1996).

Studies carried out to find the antigen which could induce the highest immune response in gilthead sea bream showed that M-like protein extracted from *E. seriolicida*/*L. garvieae* enhanced humoral immune response with high antibody titre and stimulated cell-mediated immune response, increasing phagocytosis, when compared with controls and formalin-killed cells (FKC) (Salati *et al.*, 2005). These results confirm that protection can be induced by capsular material of the bacterial cell, as suggested by Ravelo *et al.* (2003) and, particularly, that the right immunogen is located in the cell wall matrix of the bacterium which is suitable for biotechnological vaccine application. This combined with direct prophylaxis and immunoprophylaxis is an area that will help improve control of the disease.

Control, treatment and epizootiology

Stocking density of fish in net pens is directly related to the mortality caused by enterococcal infection. Mizuno, cited by Kusuda (1990), reported that yellowtail net pens with less than 1.6 kg m^3 had no mortality caused by enterococcosis/lactococcosis. However, the bacterium is in the water and an outbreak of infection has always been possible, particularly in the past, when frozen wild fish used as feed were likely infected with *E. seriolicida*/*L. garvieae*. Moreover, it is very difficult to control the disease in an intensive fish farm. *Lactobacillus plantarum* and/or *Bacillus coagulans* do not control the growth of an invasion by *E. seriolicida*/*L. garvieae* (Kusuda, 1984), while the oral administration of peptidoglycan derived from *Bifidobacterium thermophilus* seems to enhance the resistance of yellowtail against the disease (Itami *et al.*, 1996). Recently, protection induced by the use of probiotic bacteria has shown partial good results in controlling *E. seriolicida*/*L. garvieae* infection in rainbow trout (Gatesoupe, 2008; Vendrell *et al.*, 2008). Furthermore, the protective effect of bacteriophages administered orally to prevent experimental infection seems to be useful in controlling the disease (Nakai *et al.*, 1999). However, the application has not been developed in the field.

In the past, the therapy for the disease was performed using sodium nifurstyrenate, which was more active under *in vitro* and *in vivo* conditions than aminobenzyl penicillin, chloramphenicol and tetracycline (Kashiwagi *et al.*, 1977a,b). The use of erythromycin has shown improved therapy for naturally infected cultured yellowtail (Shiomitsu *et al.*, 1980). Erythromycin and spiramycin are widely used to control the disease, but there is evidence of drug resistance after ten *in vivo* serial passages in the presence of antibiotics (Kusuda and Onizaki, 1985). However, a recent study on strains isolated from rainbow trout suggests that erythromycin could be used to control the disease in trout (Vendrell *et al.*, 2008). Josamycin is considered a useful chemotherapeutant, because there are no reported side effects on fish and it does not affect its intestinal flora (Kusuda and Takemaru, 1987; Takemaru and Kusuda, 1988a). Results of *in vitro* tests on *E. seriolicida*/*L. garvieae* susceptibility to antimicrobials showed that the most efficient one was nitrofurantoin, followed by cefotaxine and norfloxacin (Vianni and Làzaro, 2003). In conclusion, josamycin and erythromycin are, to date, curative under field conditions at the beginning of the infection; then, some procedures such as stocking density reduction, excluding the use of natural feed and improvement of hygiene are also necessary. Moreover, the indiscriminate use of chemotherapeutants should be avoided because of the appearance of drug-resistant strains (Takemaru and Kusuda, 1988b,c, 1990). Since chemotherapy appeared to be poorly effective, early detection of new cases, based on rapid diagnostic methods, and direct and indirect prophylaxis are the only ways to face the disease.

Experiments on passive immunization of rainbow trout show that a high level of

protection can be obtained using mammalian antistreptococcal antibodies by injection. However, protection is limited to one month after immunization (Akhlaghi *et al.*, 1996). Studies on active immunization against the disease began in the 1980s using formalin-killed and/or heat-killed cells, detoxicated extracellular products, administered by injection, oral and/or spray methods, which provided only partial protection in marine fish (Iida *et al.*, 1981; Kusuda, 1983). It has also been reported that toxoid-enriched *Enterococcus* sp. bacterins induce a strong degree of protection in turbot that lasts for 1 year (Toranzo *et al.*, 1995). Other authors showed in trout a 6-month immunity by bacterins, without any addiction (Eldar *et al.*, 1997). Athough there are many commercial vaccines (i.e. bacterins, administered by injection) for yellowtail and for trout against this infection, the protection they induce is not high enough and lasts only a few months after administration.

Recently, good results have been obtained on protection against the disease in marine and freshwater fish (Ooyama *et al.*, 1999; Vendrell *et al.*, 2007). For example, since the start of a vaccination plan in 2005 in Oita Prefecture (Japan), losses have been limited to 5% in cultured yellowtail using a bacterin by intraperitoneal (*i.p.*) injection. At the same time, a decrease of 90% of antibiotic use was recorded (Prof T. Itami, Faculty of Agriculture, Miyazaki University, Miyazaki, Japan, personal communication, 2008). Moreover, a protective autologous vaccine administered by *i.p.* injection, starting from a bacterial strain isolated from diseased farmed trout, has been obtained in Italy (Manfrin *et al.*, 2006).

Conclusions and recommendations for future studies

E. seriolicida/*L. garvieae* is an important pathogen in aquaculture, causing outbreaks that significantly affect production. Moreover, historically, cultured fish were not considered important vectors of bacterial pathogens to humans. This situation is now changing and some pathogens of aquatic and/or cultured species, such as *S. iniae*, *Mycobacterium* spp., *Vibrio* spp. and *Edwardsiella tarda*, are reported to cause illness or disease in humans. Until the 1990s, there was little information on *E. seriolicida*/*L. garvieae* infection in humans (Elliot *et al.*, 1991). Recently, some studies on the bacterium have reported that it causes disease in humans, characterized by septicaemia with liver abscess, infective endocarditis, acute cerebral infarction and intestinal disorders (Mofredj *et al.*, 2000; Wang *et al.*, 2007; Li *et al.*, 2008). In the latter case, a correlation was suggested between seasonal aquaculture outbreaks and the consumption of raw fish, which resulted in human infection.

Therefore, a fast and sure diagnosis is needed. In the near future, real-time PCR, which enables both detection and quantification of a specific sequence in a DNA sample, and being highly sensitive, should be developed and applied to detect rapidly genes of such an important pathogen for fish and man.

Nowadays, the only possible prevention against this disease on the farm is a correct direct prophylaxis, with the hope that research in immunoprophylaxis will be successful as soon as possible. Then, to control the disease in the field, a more effective vaccine with well-defined constituents (i.e. proteins) is necessary, which is easier to produce (i.e. biotechnologically) and apply (i.e. orally with feed) on fish farms.

Streptococcus spp. (*S. iniae*, *S. agalactiae* and *S. dysgalactiae*)

Introduction

Streptococcal infection is also known as red boil disease. Bacteriology data show that the disease is caused by different species of *Streptococcus*; here, *S. iniae* is principally described but, as a cause of disease, *S. agalactiae* and *S. dysgalactiae*, and also

S. faecium, *S. faecalis* and unclassified *Streptococcus* sp., are reported.

The disease agent

S. iniae is a 0.3–0.5 μm Gram-positive, facultatively anaerobic coccus, which mostly occurs in long chains (Fig. 10.6). The bacterium grows very well on brain–heart infusion agar, nutrient agar, blood agar and Todd–Hewitt agar. The main differences compared with *E. seriolicida*/*L. garvieae* are that *S. iniae* does not grow between 10 and 45°C, 6.5% NaCl or pH 9.6 or on 40% bile agar or 0.1 % methylene blue milk.

The bacterium is Voges–Proskauer reaction-negative; it does not produce acid from sorbitol, but it does from sucrose and it is one of the few *Streptococcus* organisms producing starch hydrolysis (Kitao *et al.*, 1981; Kusuda *et al.*, 1982; Evans *et al.*, 2004). Like *E. seriolicida*/*L. garvieae*, the antigens extracted using the hot-acid method of Lancefield (1933) did not fit any of Lancefield's serotype groups; however, β-haemolysis occurred on blood agar.

Also, *S. dysgalactiae* is a Gram-positive, facultatively anaerobic coccus, which mostly occurs in long chains. The bacterium grows very well on brain–heart infusion agar, nutrient agar, blood agar and Todd–Hewitt agar. The bacterium is auto-aggregating in saline and shows negative catalase and Voges–Proskauer reactions (Nomoto *et al.*, 2004, 2006).

Different from *E. seriolicida*/*L. garvieae* and *S. iniae*, *S. dysgalactiae* is Lancefield's serotype group C when the antigens are extracted using the hot-acid method of Lancefield (1933); moreover, α-haemolysis occurs on blood agar. On the other hand, *S. agalactiae* is Lancefield's serotype group B and shows β-haemolysis.

Geographical distribution and host range

The first report on the isolation of *S. iniae* was by Pier and Madin (1976) from a

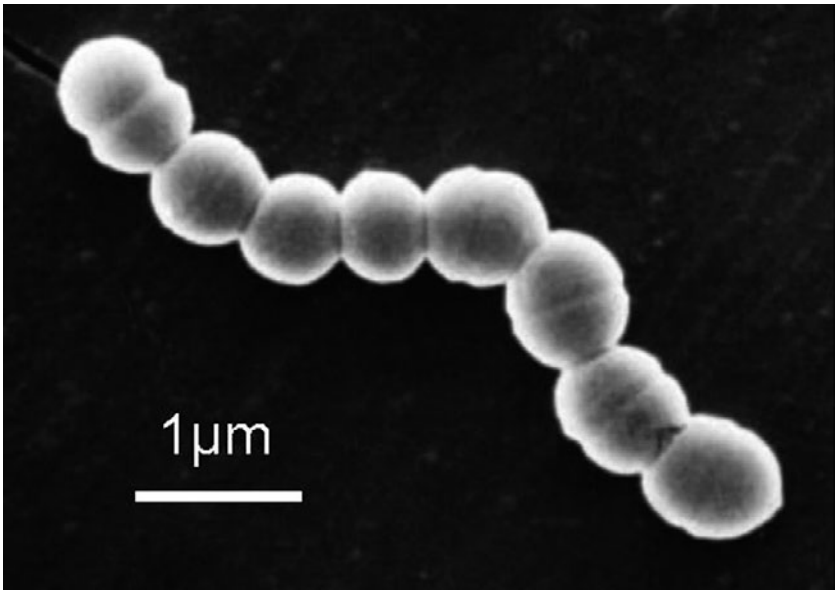


Fig. 10.6. Scanning electron micrograph of *Streptococcus iniae* cultured on BHI agar at 30°C for 24 h. From Kusuda and Salati (1993).

diseased Amazon freshwater dolphin (*Inia geoffrensis*). Subsequent large-scale epizootics caused by β-haemolytic *Streptococcus* infections were reported in Australia, Bahrain, Korea, Iran, Israel, Italy, Japan, South Africa, Spain and the USA (Boomker *et al*., 1979; Kitao *et al*., 1981; Nakatsugawa, 1983; Miyazaki *et al*., 1984; Palacios *et al*., 1993; Perera *et al*., 1994; Zlotkin *et al*., 1998b; Bromage *et al*., 1999; Eldar *et al*., 1999; Yuasa *et al*., 1999; Soltani *et al*., 2008).

There are many reports of streptococcosis in marine, brackish and freshwater fish. The first outbreak of streptococcosis was recorded by Hoshina *et al*. (1958) in rainbow trout cultured in Japan. It was later reported in the USA in a freshwater fish, the golden shiner (*Notemigonus crysoleucas*) (Robinson and Meyer, 1966) and in seawater fish such as striped mullet, menhaden (*Brevoortia patronus*), sea catfish (*Arius felis*), pinkfish (*Lagodon rhomboides*), Atlantic croaker (*Micropogon undulatus*), spot (*Leiostomus xanthurus*), stingray (*Dasyatis* sp.) and silver trout (*Cynoscion nothus*) (Wilkinson *et al*., 1973; Plumb *et al*., 1974). Other outbreaks of the disease have been reported in rainbow trout cultured in South Africa and Spain (Boomker *et al*., 1979; Palacios *et al*., 1993). *S. iniae* infection appears during the summer in periods of higher water temperatures.

The same bacterium that caused the disease in freshwater dolphin was also isolated from cultured freshwater fish such as ayu, tilapia and rainbow trout, sometimes from cultured seawater fish such as flounder and yellowtail, and from sardines (*Sardinops melanostictus*) and wild sea bream (Kitao *et al*., 1981; Nakatsugawa, 1983; Miyazaki *et al*., 1984). Experimental infection in Nile tilapia (*Oreochromis niloticus*) and in channel catfish (*Ictalurus punctatus*) showed that β-haemolytic *Streptococcus* was pathogenic to some degree in tilapia and to a lesser degree in channel catfish (Chang and Plumb, 1996). More recently, *S. iniae* has been isolated from diseased wild fish of the Red Sea, such as *Pomadasys stridens* and *Synodus variegates*, then in cultured fish such as gilthead sea bream, European sea bass (*Dicentrarchus labrax*), hybrids of tilapia (*T. nilotica*×*T. aurea*), red drum (*Sciaenops ocellatus*), rabbitfish (*Siganus canaliculatus*) and barramundi (*Lates calcarifer*) (Perera *et al*., 1994; Zlotkin *et al*., 1998b; Bromage *et al*., 1999; Eldar *et al*., 1999; Nguyen and Kanai, 1999; Yuasa *et al*., 1999; Colorni *et al*., 2002). *S. iniae* was also isolated from diseased cultured bullfrog (*Rana castesbeiana*) (Mauel *et al*., 2002). Moreover, after subcutaneous injection in murine experimental infection, it has been reported recently that the bacterium is responsible for local tissue necrosis and bacteraemia (Fuller *et al*., 2001).

S. dysgalactiae has been isolated from diseased cultured yellowtail in Japan and Siberian sturgeon (*A. baeri*) in Italy (Nomoto *et al*., 2004, 2006; Salogni *et al*., 2007).

S. agalactiae has been isolated from cultured gilthead sea bream and wild mullet (*Liza klunzingeri*) in Kuwait (Evans *et al*., 2002).

Economic importance of the disease

Since Pier and Madin (1976) first reported the isolation of *S. iniae* from a diseased Amazon freshwater dolphin (*I. geoffrensis*), the disease has spread worldwide. Large-scale epizootics caused by β-haemolytic *Streptococcus* infections have been reported, with heavy economic losses, particularly in Japan, where losses have been constant in cultured ayu (*P. altivelis*) since the 1990s. In the past decade, disease outbreaks have also caused high economic losses in other countries: losses are estimated at up to US$10 million annually in the USA and over US$100 million globally in Australia, Israel, Japan and the USA (Shoemaker *et al*., 2001). Moreover, during 2007 in Japan, *S. dysgalactiae* caused 6000 t of losses in cultured yellowtail, and all 'streptococcosis' in cultured yellowtail and flounder caused more than US$2 million economic losses (Prof Y. Takahashi, Shimonoseki University of Fisheries, Shimonoseki, Japan, personal communication, 2008).

Diagnostic methods

Streptococcosis is an infectious systemic septicaemic disease characterized by meningoencephalitis. It is easy to suspect streptococcosis according to clinical signs. However, clinical signs and gross pathology of streptococcosis caused by *S. iniae* are very similar to those of other streptococcal and enterococcal/lactococcal infections. In fact, 'streptococcosis' of fish is a disease where the most isolated pathogen is *S. iniae*, but also other frequently Gram-positive cocci such as *S. agalactiae*, *S. dysgalactiae*, *E. seriolicida*/*L. garvieae*, *L. piscium* and *Vagococcus salmoninarum* could be isolated; hence, the term 'streptococcosis' should be used to indicate the disease generally.

Clinical signs of streptococcosis are bilateral exophthalmia, listless swimming, lethargy, anorexia, dark coloration of the skin, petechiae on the inside wall of the opercula and congestion of pectoral and caudal fins and mouth (Figs 10.7 and 10.8).

The gross anatomy includes ascitis, hepatomegaly, splenomegaly and/or congestion of liver, spleen, kidney and brain. As in *E. seriolicida*/*L. garvieae* infection, the histopathological features of *S. iniae* infection have not been studied completely. It is known that histopathology reveals meningitis/meningoencephalitis and panophthalmitis, with cellular infiltration of the eye in all infected fish species examined and also in experimentally infected tilapia (*O. niloticus*), channel catfish (*I. punctatus*) and hybrid tilapia (*T. nilotica*×*T. aurea*) (Chang and Plumb, 1996; Perera *et al.*, 1998).

S. dysgalactiae infected Siberian sturgeon (*A. baeri*) shows petechiae at lips/mouth (Fig. 10.9) and fins and/or congestion of intestine, spleen, kidney and brain. The histopathological features of *S. dysgalactiae* infection have not been studied.

In order to confirm the diagnosis, it is necessary to characterize the bacterium biochemically; however, in commercial systems, the isolates are classified as 'unidentified' and/or *S. uberis*, since *S. iniae* is not included in their database. Moreover, the FA technique was used successfully to differentiate between α- and β-haemolytic cocci. A common antigen and different and characteristic antigen(s) were found in *E. seriolicida*/*L.garvieae* (α-) and *S. iniae* (β-) (Kawahara and Kusuda, 1987).



Fig. 10.7. *Streptococcus iniae* infection in cultured yellowtail (*Seriola quinqueradiata*) showing typical exophthalmia. From Kusuda and Salati (1993).



Fig. 10.8. Streptococcosis caused by *Streptococcus iniae* in ayu (*Plecoglossus altivelis*): the typical lesions of the disease showing expanded haemorrhagic anus and haemorrhage of the liver. From Kusuda and Salati (1993).

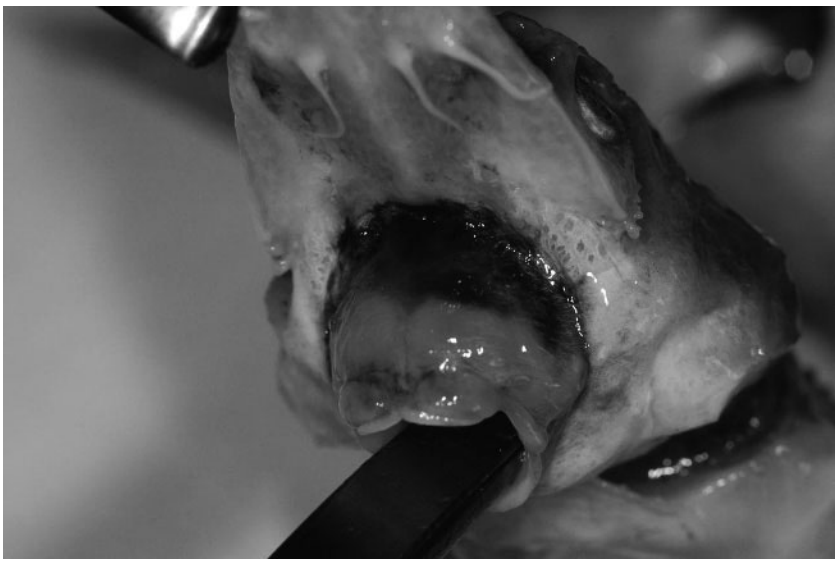


Fig. 10.9. Streptococcosis caused by *Streptococcus dysgalactiae* in Siberian sturgeon (*Acipenser baeri*): expanded haemorrhage on the mouth (photograph courtesy Drs L. Alborali and C. Salogni).

Recently, specific nested primers for the 16S–23S ribosomal DNA intergenic spacer, species-specific PCR and RAPD PCR assays have been developed for the identification of *S. iniae* (Berridge *et al.*, 1998; Zlotkin *et al.*, 1998a; Dodson *et al.*, 1999).

S. dysgalactiae diagnosis could be performed by PCR using primers designed according to the species-specific part of the 16S–23S rDNA intergenic spacer region (Hassan *et al.*, 2003b; Nomoto *et al.*, 2004).

Genomic structure and transcription

Guanine-plus-cytosine content of *S. iniae* deoxyribonucleic acid (DNA) is 35 mol %.

S. iniae capsule operon contains a homologue of the *cpsD* gene of streptococci group B (Locke *et al.*, 2007).

The mol % of guanine plus cytosine of *S. agalactiae* DNA is 32.9. The complete genome may be found on GenBank, accession number CP000114.

Pathogenesis and immunity

Streptococcosis in fish is a septicaemic disease, which causes meningoencephalitis and death in cultured fish and may become chronic. Factors that predispose to the disease are similar to those in enterococcal/lactococcal infections: these are stress, due to high fish density and suboptimal water conditions, principally high temperatures; therefore, *S. iniae* infection appears during the summer in periods of higher water temperatures. Studies performed by Zlotkin *et al.* (1998b) showed that wild and cultured fish could be infected by a single strain of *S. iniae* and suggested that wild fish could amplify the disease.

In streptococcal infections, haemolysins are known to be the exotoxins involved in pathogenicity. They are streptolysin S and SLO, but there is only one study on the haemolysin produced by *S. iniae* isolated from yellowtail. Kawahara *et al.* (1989b) demonstrated that haemolysin production was higher when the bacterium was cultured in Todd–Hewitt broth at pH 7.0, and the maximum of haemolytic activity occurred at the late-logarithmic phase and particularly after 15 h of incubation at 25°C. Little is known about the effects of fetal calf serum and bovine serum albumin on the production of haemolysin by *S. iniae*. It was suggested that the *S. iniae* haemolysin was different from streptolysin S.

It is known that other streptococcal species, such as *S. pyogenes*, *S. equi* and *S. dysgalactiae* possesses M or M-like proteins (Geyer and Schmidt, 2000; Pinho *et al.*, 2006), which are located on the surface of the bacterial cell. These proteins are considered to be the dominant virulence factor allowing antiphagocytic function and host cell attachment (Courtney *et al.*, 2006; Locke *et al.*, 2007). Baiano *et al.* (2008) demonstrated a potentially important role of fibrinogen binding *S. iniae* cell-surface proteins in avoiding phagocytic attack of peritoneal barramundi (*L. calcarifer*) macrophages. Moreover, capsule mutants were less resistant to phagocytosis than wild-type strains, showing the importance of capsule for successful infection (Miller and Neely, 2005).

Regarding the studies on the immunity of *S. iniae* infection in yellowtail, Sako (1992a) demonstrated that fish recovered from experimental infection showed a faster decrease of viable bacterial number in blood, spleen and kidney when reinfected, while an increase of bacterial cell numbers was not observed in the brain; these data suggested an acquired immunoresistance subsequent to reinfection. Sakai *et al.* (1987) demonstrated agglutinating antibodies in rainbow trout vaccinated by *i.p.* injection with a formalin-killed *S. iniae* cell preparation. However, agglutinating antibodies were not detected in fish immunized by immersion method using the same preparation. Other studies by Sakai *et al.* (1989) were performed to clarify the role of the bactericidal activity and the phagocytic activity of leucocytes from rainbow trout immunized with the formalin-killed cell preparation. Sera from fish immunized by immersion or by *i.p.* injection did not show increased bactericidal activity, while the phagocytic activity of the leucocytes increased in the vaccinated rainbow trout. The bacterial clearance in the kidney, spleen and blood was faster in both

groups of immunized fish in comparison with the controls. However, rainbow trout immunized by *i.p.* injection showed higher activities than the fish immunized by the immersion method. These studies suggest that cellular immunity plays an important role in the defence against the bacterium.

There are few studies on *S. agalactiae* as fish pathogen; however, regarding the way of infection, it has been demonstrated recently that alpha protein promotes *S. agalactiae* invasion of epithelial cells by interactions with host cell glycosaminoglycans (Baron *et al.*, 2004, 2007).

Moreover, experimentally infected Nile tilapia (*O. niloticus*) injected with *S. agalactiae* showed skeletal deformities and high mortalities of their offspring (Pasnik *et al.*, 2007).

Control, treatment and epizootiology

It is difficult to control streptococcosis because it appears when fish are stressed by poor water quality. Moreover, because the bacterium is ubiquitous (Sako, 1993a), it is not possible to eliminate the pathogen from the fish or the environment, and thus reinfection is possible. The *in vitro* activity of many antimicrobial agents shows that penicillins, cephalosporins, rifampicin, bacitracin, sodium nifurstyrenate and diaminopyrimidines are effective and that the susceptibility of *S. iniae* to these antibiotics is higher than that of *E. seriolicida*/*L. garvieae* (Sako, 1993b). In the field, erythromycin is used in chemotherapy, but spiramycin and doxycycline are also used. To control the disease in cultured rainbow trout, Kitao *et al.* (1987) showed that erythromycin administered by the oral route with the feed is effective and that *S. iniae* is sensitive to the antibiotic in both *in vitro* and *in vivo* tests.

Also, recent studies on the treatment of the disease showed good experimental results using oxytetracycline and amoxicillin (Darwish *et al.*, 2002; Darwish and Ismaiel, 2003).

There are few studies on the vaccination of fish against *S. iniae* infection. Sakai *et al.* (1987) showed the efficacy of the formalin-killed *S. iniae* cell preparation when administered by immersion or by *i.p.* injection in rainbow trout and challenged *i.p.* with live bacteria. Adjuvant does not improve protective immunity. Moreover, in farmed rainbow trout, formalin-killed *S. iniae* injected *i.p.* showed specific antibody production and protection for at least 4 months under both laboratory and field conditions (Eldar *et al.*, 1997). In yellowtail, Sako (1992b) immunized fingerlings by immersion, oral and *i.p.* injection methods; however, only vaccination by injection was effective in preventing the disease in cultured yellowtail. Recently, a study by Buchanan *et al.* (2005) suggested the use of mutant *S. iniae* without the virulence factor phosphoglucomutase to stimulate a protective immune response as a live attenuated vaccine useful for aquaculture.

Recently, results of studies on experimental vaccination of cultured tilapia, using an *S. agalactiae* bacterin preparation with adjuvant, have been reported (B. Sheehan, personal communication at the 'Managing *Streptococcus* in Warmwater Fish' Symposium, Veracruz, Mexico, 25 September 2009). To date, there are no studies on experimental vaccination of fish against *S. dysgalactiae*.

Conclusion and recommendation for future studies

Regarding reared fish, further studies on vaccination against *S. iniae* infections should examine the ways to deliver the vaccine, the length of protection and the preparation and production of antigen(s). Since enterococcal infection is the principal disease affecting cultured yellowtail, and since streptococcosis could be one of the most common diseases in many species of reared fish, the production of vaccines is of commercial interest. Consequently, this would lead the way to the study and development of new biotechnological vaccines or synthetic immunogenic substances.

Historically, cultured fish have not been considered important vectors of

human pathogens. This situation is now changing and there are some reports on *S. iniae* as a cause of illness or disease in humans: soft-tissue infections and discitis have been reported in humans (Fuller *et al.*, 2001; Koh *et al.*, 2004). The first case regarding *S. iniae* infection in humans was recorded by Weinstein *et al.* (1997): during 1995–1996 there was an epidemic in Toronto (Canada) in patients handling fresh whole fish. The results of a study performed to determine whether or not *S. iniae* isolates from fish and human were different showed that a single strain contained the virulence factor important for both humans and fish (Weinstein *et al.*, 1997; Press *et al.*, 1998). This means that there is a real possibility of transmission of *S. iniae* by improper manipulation of infected fish and a potential hazard to fish farmers, fish processors and people preparing fishmeals.

S. agalactiae originally was identified as a cause of bovine mastitis, and this disease is believed to involve biofilm formation. Moreover, it is the most common cause of sepsis (blood infection) and meningitis in newborns; it is also a frequent cause of newborn pneumonia. Nevertheless, it is still sometimes isolated from 'streptococcosis' infected fish.

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11 Mycobacteriosis and Nocardiosis

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Mycobacteriosis

Introduction

The discovery of the tubercle bacillus by Robert Koch in 1882 stands as the most important contribution to the study of tuberculosis in any host. Before the end of the 19th century, piscine mycobacteria had been described by a group of French scientists (Bataillon *et al.*, 1897). They reported that *Mycobacterium piscium* (a name which is now obsolete; Van Duijn, 1981) was a pathogen of diseased carp (*Cyprinus carpio*). Since that time, much research on mycobacterial diseases in fish has been carried out.

Von Betegh first reported mycobacteriosis in marine fish in 1910. *M. marinum* was isolated and described in 1926 by Aronson as the cause of tuberculosis-like lesions in the liver, spleen and kidney of tropical coral fish kept in an aquarium in Philadelphia (USA). This organism was thought initially to infect only marine fish, but it has since been isolated from freshwater species and humans (Barrow and Hewitt, 1971; Van Duijn, 1981). In 1953, *M. fortuitum* was another acid-fast bacillus found repeatedly in diseased neon tetra (*Paracheirodon innesi*), although the taxonomic identification of the pathogen was later described by Ross and Brancato (1959).

Many acid-fast bacteria have been recorded as aetiological agents causing mycobacteriosis in fish. However, some of them were synonymous with *M. chelonei*, *M. fortuitum* or *M. marinum*, and some species names were not considered valid by the International Working Group on Mycobacterial Taxonomy. These included *M. piscium*, *M. salmoniphilum*, *M. anabantid* and *M. platypoecilus* (Thoen and Schliesser, 1984; Dalsgaard *et al.*, 1992).

The disease agent

Mycobacteriosis in fish can be caused by several distinct species of *Mycobacterium*. This genus is a member of the order Actinomycetales and family Mycobacteriaceae. Fish mycobacteria can grow in several types of media (Table 11.1). Identification can be made using a number of criteria, including acid-fastness, growth rate, pigment production, colonial morphology and homogeneity of a suspension of the organism in a liquid medium. However, the definitive identification of each species is based on specific biochemical test reactions (Table 11.2), the deoxyribonucleic acid (DNA) level and the guanine-plus-cytosine content. *Mycobacterium* spp. are mostly resistant to conventional antibiotics. This resistance can be used as a tool for taxonomic purposes. Sero-agglutination

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Viral, Bacterial and Fungal Infections, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

tests are particularly valuable in identifying strains of *Mycobacterium* (Sato, 1962). It is, however, recommended that the cultures be submitted to a reference laboratory for final identification.

Morphologically, *Mycobacterium* are pleomorphic, acid-fast, non-motile, non-sporulated, Gram-positive, non-branching rods (0.2–0.6μm in diameter and 1.5–3.0μm long). They are slender in shape and can be beaded or barred. They are seen occasionally as filaments up to 10μm in length (Van Duijn, 1981). *Mycobacterium* found in diseased fish can be divided into two groups, one showing a slow growth rate and another that can grow rapidly and requires less than 5 days for culture.

An important staining characteristic of *Mycobacterium* is its resistance to acid decolorizing agents. Several staining procedures are available for demonstrating acid-fast bacilli, but the Ziehl–Neelsen or Kinyoun techniques with basic fuchsin are most widely used (Kubica, 1984).

There are several species of *Mycobacterium* known to cause disease in fish. *M. marinum* was isolated from coral fish (Aronson, 1926), *M. fortuitum* was named and described in 1938 by Cruz and found infecting diseased neon tetra (Ross and Brancato, 1959), *M. chelonei* was isolated from salmonids (Arakawa and Fryer, 1984), Atlantic salmon (*Salmo salar*) (Bruno *et al.*, 1998) and *M. neoaurum* from Chinook salmon (*Oncorhynchus tshawytscha*) (Backman *et al.*, 1990). *M. simiae* and *M. scrofulaceum* were first reported from black acara (*Cichlasoma bimaculatum*) and Pacific staghorn sculpin (*Leptocottus armatus*), respectively (Lansdell *et al.*, 1993). *M. abscessus*, previously considered a subspecies of *M. chelonae*, was elevated recently to species status (Kusunoki and Ezaki, 1992), and its presence was reported in Japanese medaka (*Oryzias latipes*) (Teska *et al.*, 1997). *M. avium* was isolated in dwarf cichlid (*Apistogramma cacatuoides*) raised in aquariums. *M. gordonae* was detected in three-spot gourami (*Trichogaster leeri*), angelfish (*Pterophyllum scalare*) and cardinal tetra (*Paracheirodon axelrodi*) (Lescenko *et al.*, 2003). Recently, a number of new species have been identified as being responsible for microbacterial infection in fishes. These include *M. montefiorense*, *M. haemophilum* and *M. peregrinum* (Levi *et al.*, 2003; Whipps *et al.*, 2007; Aranaz *et al.*, 2008). In addition, two novel species, *M. pseudoshottsii* and *M. shottsii*, were reported in striped bass (*Morone saxatilis*) in Chesapeake Bay, USA (Rhodes *et al.*, 2003, 2005). A review of the aetiology of mycobacteriosis including some provisional species was well presented by Gauthier and Rhodes (2009). However, the species of *Mycobacterium* most commonly isolated from fish are *M. marinum* and *M. fortuitum*.

Table 11.1. Media used to culture *Mycobacterium* sp.

Media	References
Bacto-Lowenstein medium (BLM)	Anderson *et al.* (1987) Shamsudin *et al.* (1990)
Dubos agar	Abernethy and Lund (1978)
Dorset's egg agar	MacKenzie (1988)
Dubos blood agar	Giavenni *et al.* (1980)
Harold's egg medium	Wolke and Stroud (1978)
International Union against Tuberculosis Medium (IUTM) medium	Giavenni *et al.* (1980)
Lowenstein–Jensen medium	MacKenzie (1988)
MacConkey agar	Shively *et al.* (1981) Arakawa and Fryer (1984)
Middlebrook 7H10 agar	Giavenni *et al.* (1980)
Mycobacteria 7H11 agar	MacKenzie (1988)
Ogawa egg medium	Shamsudin *et al.* (1990)
Petrignani's medium	Hedrick *et al.* (1987)
Petroff egg agar	Giavenni *et al.* (1980)
Potassium-tellurite agar	Arakawa and Fryer (1984)

Table 11.2. Characteristics of *Mycobacterium marinum* and *Mycobacterium fortuitum* (from Tsukamura, 1966).

Character	*M. marinum*	*M. fortuitum*
Colony morphology	Rough	Smooth
Pigmentation	+	–
Photochromogeneity	–	–
Growth rate	Rapid	Rapid
Catalase	+	+
$NaN0_3$ reduction	+	+
Third-day arylsulfatase	–	+
Second-week arylsulfatase	+	+
Salicylate degradation	–	+
PAS degradation	–	+
Ammonium hydroxide (NH_3OH) resistance	–	+
Picric acid tolerance (0.1%)	–	+
Picric acid tolerance (0.2%)	–	+
Growth at 28°C	+	+
Growth at 37°C	+	+
Growth at 45°C	–	–
Growth at 52°C	–	–
Acetamidase	–	+
Benzamidase	–	+
Urease	+	+
Isonicotinamidase	–	+
Nicotinamidase	–	–
Pyrazinamidase	–	+
Allantoinase	+	+
Succinamidase	–	–
Utilization of organic acids as sole carbon source		
Acetate	–	+
Citrate	–	+
Succinate	–	+
Maltate	–	+
Pyruvate	–	+
Benzoate	–	–
Malonate	–	–
Fumarate	–	+
Acid from:		
Glucose	–	+
Mannose	–	+
Galactose	–	–
Arabinose	–	–
Xylose	–	–
Rhamnose	–	–
Trehalose	–	+
Inositol	–	–
Mannitol	–	–
Sorbitol	–	–
Utilization of carbohydrates as sole carbon source		
Glycerol	+	+
Glucose	–	+

(*Continued*)

Table 11.2. *Continued*

Character	*M. marinum*	*M. fortuitum*
Fructose	–	+
Sucrose	–	–
Mannose	–	+
Galactose	–	–
Arabinose	–	–
Xylose	–	–
Rhamnose	–	–
Trehalose	–	+
Raffinose	–	–
Inositol	–	–
Mannitol	–	+
Sorbitol	–	+
Ethanol	–	+
Propanol	–	+
Propylene glycol	–	–
1,3-Butylene glycol	–	+
2,3-Butylene glycol	–	+
Utilization of nitrogen compounds as sole nitrogen source		
L-Glutamate	+	+
L-Serine	–	+
L-Methionine	–	+
Acetamide	–	+
Benzamide	–	–
Urea	+	+
Pyrazinamide	+	+
Isonicotinamide	–	+
Nicotinamide	–	+
Succinamide	+	+
Nitrate	+	+
Nitrite	–	+
Utilization of nitrogen compounds as sole nitrogen and carbon source		
L-Glutamate	–	+
L-Serine	–	–
Glucosamine	–	+
Acetamide	–	+
Benzamide	–	–
Nicotinamide	–	–
Monoethanolamine	–	+
Trimethylene diamine	–	+

$NaN0_3$, sodium nitrate; PAS, periodic acid-Schiff.

Geographical distribution and host range

Mycobacterium is ubiquitous in water and sediment and so mycobacteriosis in fish populations continues to be documented worldwide, and no country can claim that this long-recognized bacterial fish disease has been eradicated. Some 167 species of both freshwater and salt-water fish have been reported as hosts for this disease and it has been described from a wide variety of aquarium (Aronson, 1926; Besse, 1949; Nigrelli and Vogel, 1963; Giavenni *et al.*, 1980) and food fish (Alexander, 1913; Johnstone, 1913; Sutherland, 1922; Wood and

Ordal, 1958; Hublou *et al.*, 1959; Parisot and Wood, 1960; Lawhavinit *et al.*, 1988; Chinabut *et al.*, 1990; Hatai *et al.*, 1993). Mycobacteriosis has also been reported in freshwater prawn (*Macrobrachium rosenbergii*) (Brook *et al.*, 1986) and is widespread in all penaeids (Lightner, 1996).

As mycobacteriosis is a subacute to chronic disease, it seems likely that fish maintained in aquaria will show a higher incidence of this disease than cultured or wild species, because aquarium fish are often kept for long periods of time compared with fish raised for commercial purposes.

Wild stocks of fish have been reported to suffer from mycobacteriosis, including cod (*Gadus morhua*) (Alexander, 1913); halibut (*Hippoglossus hippoglossus*) (Sutherland, 1922); striped bass (*M. saxatilis*) (Aronson, 1926); North-East Atlantic mackerel (*Scomber scombrus*) (MacKenzie, 1988); and yellow perch (*Perca flavescens*) (Daoust *et al.*, 1989). In intensive fish-culture systems, mycobacteriosis was first documented in the salmon industry of the Pacific North-west (Parisot, 1958; Wood and Ordal, 1958). Mycobacterial disease has also been recorded from pejerrey (*Odonthestes bonariensis*) (Lawhavinit *et al.*, 1988; Hatai *et al.*, 1993) and snakehead fish (*Channa striatus*) (Chinabut *et al.*, 1990), which are both cultured in ponds in Japan and Thailand, respectively.

The prevalence of mycobacteriosis in aquarium fish has been reported to vary from 10 to 22% (Wolke and Stroud, 1978; Santacana *et al.*, 1982), while that of infected fish in natural populations varies from 10 to 100% (Abernethy and Lund, 1978; Sakanari *et al.*, 1983; Hedrick *et al.*, 1987; Lawhavinit *et al.*, 1988; MacKenzie, 1988). There appears to be no bias towards the sex of the fish in the prevalence of mycobacteriosis, but the severity of the infection is apparently related to age (Abernethy and Lund, 1978; MacKenzie, 1988).

Disease outbreaks in cultured fish appear to be related to management factors, such as the quality and quantity of nutrient and water supplied, and the stocking density. Poor management results in abnormal stress and a reduction in the normal resistance of the host.

Economic importance of the disease

Infections in some wild fish populations might be as high as 15% (Parisot and Wood, 1960), whereas infections in intensively cultured fish might reach 100% (Smith, 1996). Giavenni *et al.* (1980) examined approximately 300 marine and freshwater tropical fish for mycobacteriosis and found that the mortality rate among fish was between 5 and 10%. Striped bass (*M. saxatilis*) raised in California were infected with *M. marinum*, resulting in 50% losses (900 yearling), and those that survived had chronic infections (Hedrick *et al.*, 1987). Snakehead (*C. striatus*), a valuable freshwater farmed fish in Thailand, has a mortality rate which can be as high as 20%. Fish infected with mycobacteriosis, particularly food fish, are of no market value as they are unfit for human consumption. It has been reported that companies raising striped bass in the USA have condemned several million dollars worth of fish due to mycobacterial infections (Smith, 1996). Mycobacteriosis outbreaks have also been reported in cultured sea bass (*Dicentrarchus labrax*) in Israel, with a 100% prevalence of *Mycobacterium* infection and over 50% mortality (Colorni, 1992).

Mycobacteriosis is common in ornamental fish, with many being susceptible to the disease (Nigrelli and Vogel, 1963). For instance, Siamese fighting fish (*Betta splendens*), an economically important fish for export from Thailand, is particularly susceptible to *Mycobacterium* infections (Adams *et al.*, 1995). Mortality can be higher than 30% during the disease outbreak. In the period from 2002 to 2005, between 29.9 and 46.8% of samples of freshwater and marine ornamental fish imported to Italy were infected with *Mycobacterium* spp. (Zanoni *et al.*, 2008).

Diagnostic methods

Clinical signs

Piscine mycobacteriosis is a slowly developing chronic disease, which may take 2 or more years for the number of organisms to

grow to readily detectable numbers (Ashburner, 1977). Most species of fish may manifest few or no external signs of the disease. However, in advanced stages, emaciation, cachexia, exophthalmia, lordosis, haemorrhagic and dermal ulcerative lesions or loss of scales may be observed. Other signs of infection can be seen in the gills, which are paler than normal and show thickened areas on some filaments. Small lesions may be observed around the mouth and vent. Changes in cutaneous pigmentation include a fading of normal colour in aquarium fish (Snieszko, 1978) and a bright coloration in salmonids (Ross, 1970). Affected fish generally exhibit lethargic behaviour, floating impassively on the surface of the water, and with concurrent anorexia.

Gross pathology

The gross signs of infection include an enlargement and softening of spleen, kidney and liver. Sometimes, a number of greyish-white nodules are seen peppered throughout these organs. In severe cases, almost all the visceral organs are swollen and fused by whitish membranes around the mesenteries, and fluid accumulates in the peritoneal cavity (Chinabut *et al.*, 1990).

Diagnosis

Diagnosis of mycobacteriosis depends on clinical and histological signs and identification of the bacterial pathogen. Mycobacteriosis in fish is normally localized in the skin and internal organs and appears as nodular structures with a typical granulomatous pattern. Smears from scrapings of the cut surface of spleen and kidney tissues are air-dried and stained with the Kinyoun modification of the Ziehl–Neelsen stain. The stained slides are examined with an ordinary light microscope for acid-fast bacilli, which appear as coccoidal or bacillary rods (1–3μm in length). Fluorescing dyes, such as auramine or rhodamine, are recommended with either blue or ultraviolet and a microscope equipped with special filters (e.g. EY-455 and EO-530). Acid-fast bacilli are seen as short, yellow-fluorescing rods. An immunocytochemical method using the avidin–biotin complex (ABC) was recommended by Gómez *et al.* (1993) to demonstrate the small number of mycobacteria in the section of affected tissues. Monoclonal antibodies have been used as specific probes in immunohistochemistry and in the diagnosis of archival tissues (Puttinaowarat *et al.*, 2000). Conventional diagnosis of mycobacterial infection requires the isolation and identification of the organisms from skin lesions, spleen or kidney. The procedures for isolating mycobacteria are the same as those used for other bacteria. Specimens must be treated to kill contaminants (Fig. 11.1).

As conventional diagnosis is time-consuming due to the slow growth of the bacteria, alternative techniques have been developed, including enzyme-linked immunosorbent assay (ELISA), which is a powerful serological assay widely used in clinical diagnosis and applied successfully to detect mycobacterial antigens in exotic animals (Thoen *et al.*, 1980). Consequently, the ELISA technique has resulted in the rapid development of monoclonal antibodies to facilitate serological assays, for both diagnostic and taxonomic purposes (Tsang *et al.*, 1984; Arakawa *et al.*, 1986; Adams *et al.*, 1995, 1996; Blackwell, 1998; Puttinaowarat *et al.*, 2000).

Polymerase chain reaction (PCR) is a popular molecular method used for diagnosis. Various DNA sequences of *Mycobacterium* are targeted for amplification, including 16S rRNA gene, internal transcribed spacer (ITS) rDNA, heat shock protein (*hsp65*) gene and superoxide dismutase gene (Knibb *et al.*, 1993; Zolog and Philippi-Schulz, 1994; Talaat *et al.*, 1997; Puttinaowarat *et al.*, 2002; Ucko *et al.*, 2002; Whipps *et al.*, 2003). The 16S rRNA gene is the most popular target as it is highly conserved in mycobacteria. It is often utilized as a genus-specific probe in first-round PCR, followed by more specific application such as a second-round, species-specific PCR, restriction enzyme analysis or stringency hybridization assay.

In situ hybridization is a useful method for identifying the pathogens in archival samples (Arnoldi *et al.*, 1992; McNicol and Farquharson, 1997). This technique combines

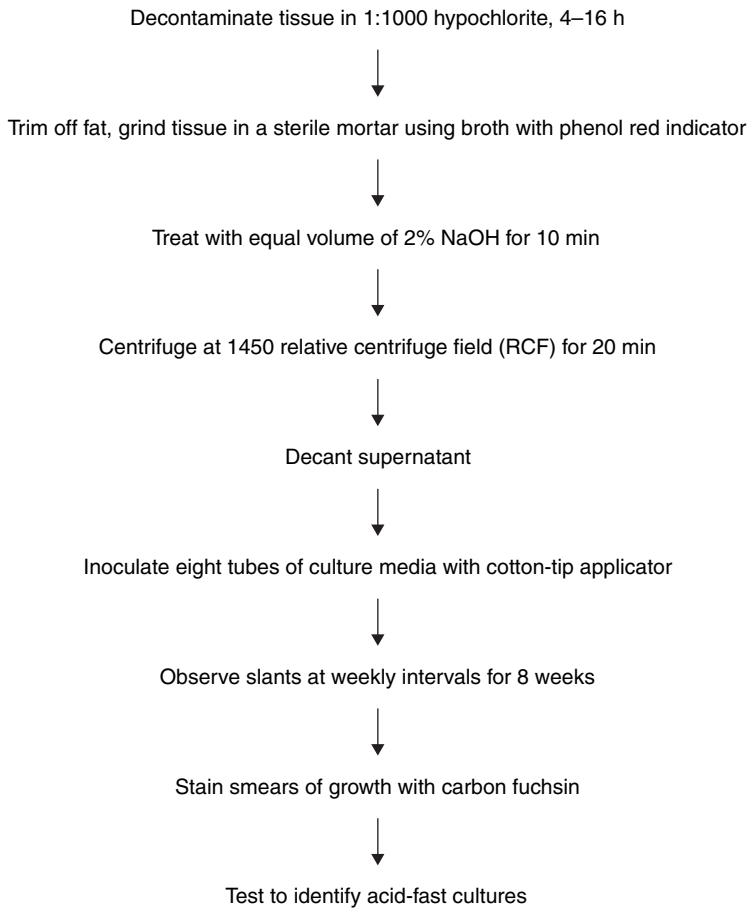


Fig. 11.1. Isolation procedure for mycobacteria (Thoen and Schliesser, 1984). NaOH, sodium hydroxide.

the advantage of microscopic investigation with molecular technology, providing pathological information and specific identification. Radioactive and non-radioactive labelling can be used to probe the DNA. The main advantage of the radioactive method is that very small amounts of the target molecules can be detected with a high signal. However the method is not widely used because of the limited stability of the radioactive probes, short half-life, long exposure of time required and the added problems of handling and disposal of radioactive materials. As an alternative, non-radioactive assays generate an almost equivalent signal through an enzymatic reaction with a chemiluminescent or chromogen substrate. The method also has the advantage of providing a long shelf life.

Physico-chemical methods have also been reported. For example, pyrolysis mass spectrometry (PyMS) is a versatile method for mycobacteria identification and characterization, as it is a rapid and accurate method requiring minute samples (Sisson *et al.*, 1992; Freeman *et al.*, 1993; Puttinaowarat *et al.*, 1999).

Histopathology

Mycobacteriosis lesions in fish are normally localized in the skin (Fig. 11.2) and internal



Fig. 11.2. Subcutaneous granuloma in Siamese fighting fish infected with *Mycobacterium*, ×130 (haematoxylin and eosin (H & E) stain).

organs (Fig. 11.3) and consist of nodular structures with characteristic focal granulomas. These are composed of centrally located epithelioid cells and macrophages. The sizes of the granulomas vary from 80 to 500 μm (Sakanari *et al.*, 1983). Mycobacteriosis can be divided into subacute and chronic forms (Wolke and Stroud, 1978). In the subacute form, there is a diffuse distribution of reticuloendothelial cells and macrophages (Fig. 11.4), with large caseous necrotic areas. Acid-fast bacilli are found scattered among the reticuloendothelial cells and within the cytoplasm of phagocytic macrophages. The chronic proliferative form is characterized by the production of both hard and soft granulomas. Soft granulomas have four distinguishable layers. In the centre is an area of caseous necrosis, with or without nuclear debris, surrounded by a layer of spindle-shaped epithelioid cells. The third layer contains highly eosinophilic, flattened, epithelioid cells, while the outermost layer is composed of fine fibrous connective tissue encircling to form a thin capsule. Hard granulomas are composed of epithelioid cells encapsulated by fibrous connective tissue. The layer of epithelioid cells closest to the zone of caseation and fibrous capsule may not be seen in all granulomas. Noga *et al.* (1989) have indicated that cells participating in the chronic inflammatory response to mycobacteria may not be derived from mononuclear phagocytes. As the identification of these cells was uncertain, they proposed the name, chronic inflammatory foci (CIF), instead of tubercle granuloma.

Calcification in the caseous necrotic centre occurs in more chronic infections (Majeed *et al.*, 1981; Van Duijn, 1981; Anderson *et al.*, 1987). Melanization and vacuolation may be found surrounding the cutaneous granulomas (Noga *et al.*, 1990). Such granulomas are all of the soft tubercle type, occurring in the stratum spongiosum of the dermis (Anderson *et al.*, 1987). Melanomacrophage centres are close to the granulomas in the spleen and kidney of infected fish and giant cells are not typically found in piscine mycobacteriosis (Wolke and Stroud, 1978; Anderson *et al.*, 1987). However, they may be found at the early stage of the granulomatous formation (Timur and Roberts, 1977).

At the early stages in the development of the disease, the spleen, kidney and liver are the primary target organs. In severe cases, a number of granulomas spread to almost

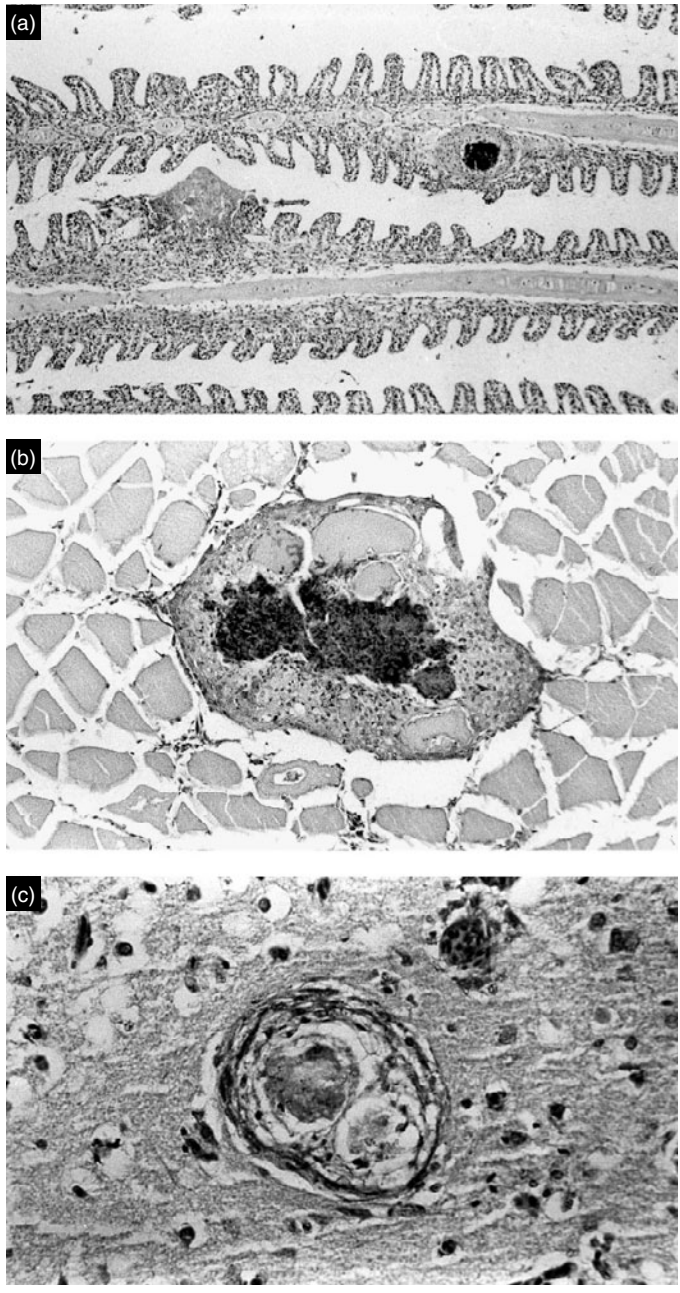


Fig. 11.3. Tubercle granuloma in snakehead fish infection with *Mycobacterium*: (a) gills, ×130; (b) muscle, ×250; and (c) brain, ×500 (H & E stain).

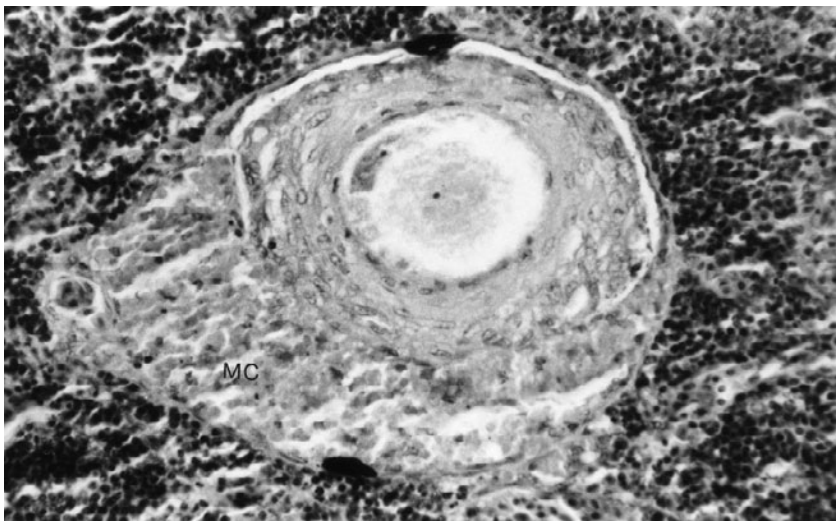


Fig. 11.4. Macrophage with lipofuscin in the cytoplasm accumulating at the edge of the granuloma to form a melanomacrophage centre in an infected fish, ×500 (H & E stain).

all the visceral organs and some of them fuse to form a large granuloma enclosed by loose connective tissue (Giavenni *et al.*, 1980). Acid-fast bacilli have been seen occasionally in the caseous necrotic centre and in the cytoplasm of the surrounding epithelioid cells and macrophages. These bacilli may also be found free in the swim-bladder lumen or in the cytoplasm of sloughed epithelial cells (Anderson *et al.*, 1987).

Genome structure and transcription

General features

Phylogenetic studies have revealed that *M. marinum* is related most closely to *M. ulcerans* and *M. tuberculosis*, with nucleotide identity over 97 and 85%, respectively (Tønjum *et al.*, 1998; Stinear *et al.*, 2000; Devulder *et al.*, 2005). Although *M. marinum* and *M. ulcerans* are closely related, they are phenotypically distinct and cause different pathologies. Divergence has been reported in two insertion sequences, IS*2404* and IS*2606* for the two bacteria, which are not found in *M. marinum* but are present in *M. ulcerans* (Stinear *et al.*, 1999, 2000).

The complete *M. marinum* genome has been published recently by the Sanger Institute (ftp://ftp.sanger.ac.uk.pub.yyy). Briefly, the genome is composed of a single circular chromosome of 6,636,827 bp with 5424 protein-coding sequences (CDS), 65 pseudogenes, 46 tRNA genes, a single rRNA operon and a 23 kb mercury-resistance plasmid (pMM23) (Stinear *et al.*, 2008). This mercury resistance could be useful as a genetic marker for epidemiological investigations.

Proteins and related gene loci

RD1 AND PROTEINS. Like all other bacteria, mycobacteria possess a secretion system to export proteins across the cytosolic membrane and the secretion system that has been of interest recently is the so-called ESX-1 secretion system. It is encoded by the genes within RD1 (region of difference 1). The ESX-1 is essential for mycobacterial pathogenesis, in particular intercellular dissemination and granuloma formation. These two traits have been identified in the most pathogenic *Mycobacterium* (e.g. *M. marinum* and *M. tuberculosis*), except for

M. ulceran, which does not appear to survive intracellular infection (Stinear *et al.*, 2007, 2008). There are 11 proteins identified in the ESX-1 secretome of *M. marinum*, three of which are CFP-10, ESAT-6 and Mh3881c (homologous to *M. tuberculosis* Rv3881c) and involve co-dependent secretion. The role of co-dependent secretion is required for intracellular growth within macrophages, which correlates with its role in inhibiting maturation of the *Mycobacterium*-containing phagosome (Xu *et al.*, 2007).

Much work has been carried out recently on the region of difference (RD) between clinical strains, *M. tuberculosis* and *M. bovis* bacillus Calmette–Guérin (BCG), in an attempt to understand the limitation of BCG. Attention was first drawn to the role of the RD region in virulence when it was realized that all BCG vaccine strains had this region missing in their chromosome (Mahairas *et al.*, 1996; Behr *et al.*, 1999; Gordon *et al.*, 1999). Three RD regions have been identified (RD1, RD2 and RD3) and all are missing from the BCG strains (Mahairas *et al.*, 1996). Also, 13 regions representing 129 open reading frames (ORFs) of the *M. tuberculosis* genome are lacking in the BCG strains (Behr *et al.*, 1999; Gordon *et al.*, 1999). The RD1 encompasses nine genes (from *Rv3871* to *Rv3879c*) in *M. tuberculosis*, while in *M. marinum* multiple genes appear to be involved in a larger RD1 region (extRD1) spanning *Mh3866–Mh3881c*. The RD1 locus encodes a number of proteins involved in virulence. Mutations in these regions lead to a loss of ESAT-6 and CFP-10 secretion (Gao *et al.*, 2004). The extRD1 genes are essential for the transmission of *M. marinum* from cell to cell among macrophages and dendritic cells. The failure of antibiotic treatment suggests strong evidence of cell-to-cell dissemination, as the bacteria are not exposed to the extracellular milieu (Gao *et al.*, 2004).

ESX-5 LOCUS AND PE/PPE PROTEINS. As well as the ESX-1 secretion system, the ESX-5 locus has been described recently in *M. marinum*, and has also been found to be conserved among a number of pathogenic mycobacteria, except *M. smegmatis* (Gey van Pittius *et al.*, 2001). It is responsible for secreting essential elements for bacterial viability (Abdallah *et al.*, 2006) including the PE/PPE proteins, so called after their characteristic proline–glutamate (PE) or proline–proline–glutamate (PPE) motifs near the N-terminus (Cole *et al.*, 1998). The PPE secretion system is also believed to be involved in the virulence mechanism of the bacterium, particularly with the spread of infection to uninfected macrophages. Although the purpose of the proteins in this group is not fully understood, it is believed that the genes that they encode co-evolved with ESX loci and expanded in the common ancestor of *M. marinum*, *M. tuberculosis* and *M. ulcerans* (Gey van Pittius *et al.*, 2006). Complete genome sequencing of *M. marinum* has revealed 281 CDS distributed around the genome encoding 27 PE, 106 PPE and 148 PE-PGRS glycine-rich proteins (Stinear *et al.*, 2008). The polymorphic GC-rich-repetitive sequence (PGRS) is a subfamily of the PE family, which contains proteins with multiple tandem repeats of a glycine-glycine-alanine (Gly-Gly-Ala) or a glycine-glycine-asparagine (Gly-Gly-Asn) motif in the C-terminal domain (Gey van Pittius *et al.*, 2006). Some *M. marinum* PE-PGRS proteins are known to be involved in the intracellular survival of the bacterium. Mutation of two PE-PGRS genes (*mag* 24 and *mag* 85) in *M. marinum* has resulted in the mutant being incapable of replication in macrophages and decreased persistence in granulomas (Ramakrishnan *et al.*, 2000).

LIPOPROTEINS. Mycobacterial lipoproteins are crucial components of the unique mycobacterial cell envelope, which protects the bacteria from environmental stress and the defences of the host (Rezwan *et al.*, 2007). Variations in the lipoprotein occur among different species of *Mycobacterium* and may reflect the different host range of each *Mycobacterium* species (Rezwan *et al.*, 2007). *M. marinum* has 87 CDS encoding lipoproteins, compared with 89 in *M. tuberculosis* (Stinear *et al.*, 2008). It has been shown that several lipoproteins in *M. tuberculosis*

are required for optimal growth of the bacterium *in vivo* (Sassetti and Rubin, 2003; Rezwan *et al.*, 2007). As lipoproteins play a role in the virulence of the bacterium by interacting with the host immune response, they may act as potential vaccine candidates. One lipoprotein is also thought to be involved in the translocation of complex lipids across the outer membrane of *M. tuberculosis* (Sulzenbacher *et al.*, 2006).

Pathogenesis and immunity

Like other virulent mycobacteria, *M. marinum* is an intracellular pathogen which survives and replicates in the host macrophages, where it prevents phagosomal maturation (Barker *et al.*, 1997; Tobin and Ramakrishnan, 2008). The ability to grow within the host macrophages is an essential virulence characteristic of pathogenic mycobacteria (Cosma *et al.*, 2003). All pathogenic mycobacteria reside inside vacuoles of the host macrophages, where they do not fuse with the lysosomes. This restriction limits the interface between the pathogen and antigen processing and presentation mechanisms of the macrophage (Russell, 2001).

Granuloma formation is the pathological hallmark of mycobacteria infection. Volkman *et al.* (2004) demonstrate that mycobacteria fail to elicit granuloma formation, despite their ability to grow inside infected macrophages. The infected macrophages produce signals directing them to aggregate into granulomas, which in turn are linked to intercellular bacterial dissemination and increased bacterial numbers. The RD1 locus encoding is also thought to have a role in the aggregation of infected macrophages and in the recruitment of additional macrophages.

The pathogenesis of *M. marinum* infection has been demonstrated in zebrafish (*Danio rerio*) (Davis *et al.*, 2002). An early immune response is detected after 1 h following injection with *M. marinum*. Macrophage phagocytosis is one of the first defence mechanisms to be mobilized by the host; however, this mechanism cannot eradicate the bacteria totally because of their ability to evade detection (Tobin and Ramakrishnan, 2008). Mycobacteria reside within macrophages, which can be seen in various tissues 1 day after infection. After 3 days, macrophage aggregations can be identified in the tissues (Davis *et al.*, 2002). After macrophage aggregations, the infected macrophages subsequently differentiate into epithelioid and multinucleated cells and form granulomas within 4 days (Bouley *et al.*, 2001; Tobin and Ramakrishnan, 2008). Granuloma formation is innate immunity as it is normally formed before the development of adaptive immunity.

Two groups of genes have been identified with granuloma formation in zebrafish. These include macrophage-activated genes (*mags*) and granuloma-activated genes (*gags*) (Davis *et al.*, 2002). The *mags* are activated once the macrophages phagocytose the mycobacteria. Once the macrophages form aggregates, the *gags* are then activated (Davis *et al.*, 2002).

At present, vaccines against fish mycobacteriosis are not commercially available. Chen *et al.* (1996) reported that vaccination of rainbow trout with extracellular products of *Mycobacterium* sp. had enhanced the level of reactive oxygen species, phagocytosis, lysozyme activity and antibody production.

The development of DNA vaccines to various intracellular fish pathogens has been studied by Boudinot *et al.* (1998) and Lorenzen *et al.* (1998). DNA vaccination is based on the introduction of plasmid DNA encoding the protective antigen into the animal tissue, where the encoded antigen is expressed and subsequently induces the host immune response. Of the potential antigens, antigen 85 complex, which is a fibronectin-binding protein conserved across *Mycobacterium*, has been a prime candidate for vaccine development. However, Pasnik *et al.* (2003) demonstrated significant specific humoral and cell-mediated immune responses in striped bass (*M. saxatilis*) vaccinated with a recombinant 85A antigen vaccine, but the fish failed to demonstrate cross-protective response after the vaccination. Pasnik and Smith (2005, 2006) also demonstrated that a DNA vaccine encoding antigen 85A conferred protective immunity in hybrid-striped bass (*M. saxatilis*×*M. chrysops*) against

M. marinum. However, duration of protection was limited to a high-dose challenge.

Control, treatment and epizootiology

Fish may be infected by ingesting feed and water contaminated with faecal material, urine or exudates from diseased animals that contain mycobacteria (Ross and Johnson, 1962). Dos Santos *et al.* (2002) suggested that contaminated inlet water was one of the sources of infection occurring in the fish farm. The sources and modes of transmission of mycobacterial infections in fish may be related to the infection of invertebrates such as arthropods (Beerwerth *et al.*, 1979), freshwater snails (Michelson, 1961) or freshwater prawns (Brook *et al.*, 1986). The entry of mycobacteria through skin and gill lesions caused by injury or parasitic infection is also possible. After the organisms enter the body, they may cause skin lesions or spread to other organs through the circulatory or lymphatic system. Malnutrition has also been suggested to be a contributing factor to susceptibility to mycobacterial infection (Belas *et al.*, 1995).

Some scientists have tried to prove that *M. tuberculosis* excreted from human patients may transform to cause disease in cold-blooded animals. However, it is now clear that such transformations do not occur (Thoen and Schliesser, 1984).

The observation of mycobacteria in the piscine ova and tubercle granulomas in the ovary wall suggests that transovarian transmission is a definite possibility. Johnstone (1913) reported that the ova of infected fish were small and markedly undeveloped and contained numerous acid-fast bacteria. A report from an Australian fish hatchery has provided evidence that *Mycobacterium* can be introduced by eggs and transmitted to the Fl generation (Ashburner, 1977). However, this observation does not confirm that ovarian transmission takes place, as the egg surface may be contaminated by peritoneal fluid containing mycobacteria (Conroy, 1964a). Chinabut *et al.* (1994) confirmed the transmission of mycobacteria in Siamese fighting fish (*B. splendens*) via transovarian passage. Acid-fast bacteria were found in the ova of diseased female Siamese fighting fish, using a fluorochrome technique.

The implementation of preventive measures in controlling chronic mycobacteriosis is particularly relevant, due to difficulties in treatment. Anderson *et al.* (1987) consider that chemotherapy is often unsuccessful. The importance of good management practices should be recognized. Fish should be obtained from farms known to be free of disease, and imported fish quarantined. Trash fish or dead fish carcasses used as a source of protein in the feed should be heated for 30 min at 76°C (Thoen and Schliesser, 1984) to kill pathogenic mycobacteria. Formalin or phenolic compounds should be used as disinfectants on farms at which mycobacteriosis infections have been recorded. Soaking nets and other equipment in 200 mg/l chlorine for 5 min was recommended, as well as disinfecting each individual worker on entering and exiting the aquacultural area (Jacobs *et al.*, 2004). Some disinfectants including 50 and 70% ethanol, benzyl-4-chlorophenol-phenylphenol and sodium chlorite are considered to be the most effective in reducing the number of *M. marinum* to zero within 1 min of contact time (Mainous and Smith, 2005). Dead fish should be destroyed by burning, or buried in quicklime. Chloramine B or T at a concentration of 10 mg/l for 24 h is recommended for bath treatment (Van Duijn, 1981). Most temperate fish become more susceptible to the disease when they are stressed by high temperature and poor water quality. A water-cooling system in fishponds during the summer may keep the disease at a low level.

Studies have shown that the efficacy of antibiotic treatment against *Mycobacterium* is variable. Some antibiotics could reduce the intensity of the mycobacterial colony; however, none could eliminate the infection successfully. Therapeutic measures, such as adding tetracycline to the water at a concentration of 30 mg/l, have been efficacious in treating acute stages of the disease (Van Duijn, 1981; Thoen and Schliesser, 1984). A combination of antimicrobials, including streptomycin, ethambutol, cycloserine, cotrimoxazole, rifampicin and tetracyclines, has been used

and thought to be effective. Among these drugs, cotrimoxazole or rifampicin with ethambutol have been used most frequently (Pattyn, 1984). Isoniazid and rifampicin have been recommended for treating mycobacteriosis in valuable exotic marine fish (Dulin, 1979). Boos *et al.* (1995) reported that treatment of mycobacteriosis in tropical ornamental fish with rifampicin was effective; however, the bacteria were still isolated from the infected host after the treatment.

Public health

An important aspect of fish mycobacteriosis is that some of the causative mycobacteria also cause skin disease in humans. Human infection with *M. fortuitum* and *M. marinum* has been widely reported. *M. marinum* infections in humans have been known since 1951 and have been described in some temperate and tropical countries, such as Sweden, The Netherlands, Belgium, the UK (Pattyn, 1984), Canada (Brown *et al.*, 1977), the USA (Mollohan and Romer, 1961) and Thailand (Bovornkitti *et al.*, 1991). These outbreaks were associated with cutaneous abrasions and exposure to swimming-pool water contaminated with *M. marinum*. Allergic dermatopathies have also been reported on the skin of aquarists handling water in which affected fish have been reared (Barrow and Hewitt, 1971; Giavenni *et al.*, 1980; Huminer *et al.*, 1986; Kullavanijaya *et al.*, 1993).

The rapidly growing *M. fortuitum* has been cultured from patients with pulmonary disease and local abscesses (Cruz, 1938). *M. chelonei* has been isolated from heterograph heart-valve transplants and lesions have also been found in synovial fluid and muscle (Blacklock and Dawson, 1979; Thoen and Schliesser, 1984).

Recommendations for future studies

Fish mycobacteriosis has been studied for more than a century but the successful vaccination of fish against mycobacteria has yet to be achieved, so emphasis should be placed on research into the development of a mycobacteriosis vaccine. The development of rapid diagnostic kits to detect *Mycobacterium* is also an urgent requirement in aquaculture.

Nocardiosis

Introduction

Nocardiosis is a disease of both salt-water and freshwater fish caused by actinomycetes of the genus *Nocardia*. Cases of piscine *Nocardia* are not as widely reported as mycobacteriosis in fish. Some nocardial infections in fish may be misinterpreted as mycobacterial disease, as they both result in similar clinical signs and gross pathology. *N. asteroides*, described by Eppinger in 1891, is considered the type species of the genus (Gordon and Mihm, 1962). Formerly, *N. farcinica* had been regarded as the type species, but this was changed due to the absence of an authentic strain in any culture collection and the paucity of references to *N. farcinica* in the literature. The first case of fish nocardiosis caused by *N. asteroides* was reported in neon tetra (*Hyphessobrycon innesi*) (Valdez and Conroy, 1963).

The disease agent

Nocardioform bacteria are identified tentatively by their rapid growth rate, which ranges from 3 to 7 days, and the characteristic small, heaped and wrinkled moist to chalky colonies. They are aerobic, Gram-positive bacteria. The morphology of *Nocardia* varies from a branched filamentous cell to a fragmented, irregularly shaped, pleomorphic or coccobacillary cell, depending on the phase of growth (Beaman *et al.*, 1988). The cell walls of *Nocardia* contain branched, hydroxylated fatty acids. The acid-fast staining property of this organism can distinguish it from other genera of actinomycete bacteria. It is difficult to differentiate between *Nocardia* and rapidly growing mycobacteria by their

physiological and morphological characteristics (Uesaka, 1956; Gordon, 1966; Tsukamura *et al.*, 1981).

The use of a number of antibiotics at suitable concentrations has provided valuable data for the classification and identification of nocardioform bacteria. These include gentamycin, kanamycin, neomycin, streptomycin, tobramycin, rifampicin, cephaloridine, novobiocin, fusidic acid and vancomycin. Sensitivity tests were found to be particularly useful for this purpose (Goodfellow and Orchard, 1974). The comparative immunodiffusion method was used by Ridell (1981) to classify *Nocardia*. Direct fluorescence antibody techniques have also provided reasonable results for the identification of *N. kampachi* (currently named *N. seriolae*) (Kusuda and Kawahara, 1987).

The various species of *Nocardia* can be differentiated by the temperature ranges at which they grow. *N. asteroides* grows at 37°C and can survive at 50°C for more than 4 h, but *N. seriolae* does not grow at 35°C (Kusuda and Taki, 1973; Kusuda, 1975).

Currently, there are only three main pathogenic species in fish; these include *N. asteroids*, *N. salmonicida* and *N. seriolae* (formerly called *N. kampachi*). Only one species, namely *N. crassostreae*, has been reported in shellfish. Phenotypic properties among these four species are illustrated in Table 11.3.

Table 11.3. Phenotypic properties among *Nocardia asteroides*, *N. salmonicida*, *N. seriolae* and *N. crassostreae* (Friedman *et al.*, 1998; Isik *et al.*, 1999; Kageyama *et al.*, 2004).

Property	*N. asteroides*	*N. salmonicida*	*N. seriolae*	*N. crassostreae*
Colour of colony	Beige	Orange	Pale yellow/pale orange	Pale yellow
Aerial hyphae	+	+	–	–
Nitrate reduction	+	+	+	ND
Decomposition of:				
Adenine	–	–	–	ND
Aesculin	+	+	+	+
Arbutin	+	+	+	ND
Casein	–	–	–	–
Elastin	–	–	–	ND
Hypoxanthine	–	–	–	–
Testosterone	+	+	–	ND
Tyrosine	–	+	–	–
Urea	–	+	–	–
Xanthine	–	–	–	–
Utilization of:				
Acetate	+	+	+	ND
Adipic acid	–	ND	ND	ND
Mannitol	+	+	–	–
Rhamnose	–	–	–	ND
Sorbitol	–	+	–	ND
Citrate	+	+	+	–
Arylsulfatase activity	–	ND	ND	ND
Growth at 37°C	+	–	–	–
Growth at 45°C	–	–	–	–
Susceptibility test:				
Imipenem	+	ND	ND	ND
Tobramycin	+	ND	ND	ND
5-Fluorouracil	–	ND	ND	–

ND, no data available.

Transmission of the disease

The routes by which infection takes place are not known. Most cases of piscine nocardiosis involve the oral cavity, but oral exposure has not been established experimentally as a primary route of infection. Sindermann (1990) suggested that farmed fish could be infected through contaminated feed. Bransden *et al.* (2000) suggested that phylloplane flora containing a high proportion of Gram-positive bacteria could introduce pathogens into the fish tanks of salmon farms via falling leaf litter of overhanging trees.

Geographical distribution and host range

Information on fish nocardiosis is very limited in comparison with mycobacteriosis. However, this does not mean that nocardial infection is less of a problem in fish than mycobacteriosis. *N. asteroides* has been reported to be the cause of granulomatous disease in neon tetras kept in aquaria in Argentina (Conroy, 1964b). Snieszko *et al.* (1964) reported nocardial infection in hatchery-reared fingerling rainbow trout (*O. mykiss*) in Leetown, West Virginia, USA, and subsequently Campbell and MacKelvie (1968) isolated *Nocardia* from brook trout (*Salvelinus fontinalis*) in Canada. Nocardiosis was also observed in cultured yellowtails (*Seriola quinqueradiata* and *S. purpurascens*) in Japan (Kubota *et al.*, 1968). Later, there was a report of *N. seriolae* infection in Japanese flounder (*Paralichthys olivaceus*) (Kudo *et al.*, 1988). An outbreak of nocardiosis was recorded in Formosa snakehead fish (Channidae) from Taiwan (Hsu *et al.*, 1987) and, more recently, a case of *N. seriolae* in China was reported in snakehead (*Ophiocephalus argus*) (Wang *et al.*, 2007). Outbreaks of this disease have also been reported in Japanese sea bass (*Lateolabrax japonicus*) and yellow croaker (*Larimichthys crocea*) in Taiwan and China, respectively (Chen *et al.*, 2000; Wang *et al.*, 2005). Nocardioform actinomycetic organisms were also isolated from many species of freshwater fish affected with epizootic ulcerative syndrome (EUS) in India (Chakrabarty and Dastidar, 1991). Other aquatic animals, including crayfish (*Austropotamobius pallipes*) and Pacific oyster (*Crassostrea gigas*), are also susceptible to nocardial infection (Friedman and Hedrick, 1991; Bower *et al.*, 1994; Friedman *et al.*, 1998).

Economic importance of the disease

Economically, nocardiosis has not been considered important because it is thought to be a chronic disease causing low mortality. However, Labrie *et al.* (2008) have suggested recently that this disease can affect marketable fish (over 100 g) and can therefore cause significant loss. Indeed, the mortality of 18-month-old snakehead (*O. argus*) infected with *N. seriolae* was reported to be up to 35% (Wang *et al.*, 2007).

Nocardial infections in marine species have been reported in yellowtail (*S. quinqueradiata*) and amberjack (*S. dumerelli*) in Japan (Shimahara *et al.*, 2008). Outbreaks also occurred in Japanese sea bass (*L. japonicus*) and yellow croaker (*L. crocea*) in Taiwan and China, with mortality rates higher than 15% (Wang *et al.*, 2005).

Diagnostic methods

As mentioned earlier, nocardiosis may be misdiagnosed as mycobacteriosis, because of the similarity between the clinical signs and gross pathology associated with the two diseases. Positive differentiation can only be made by isolation and identification of the causative agent. In addition, histological sections of the infected organs should be examined. Nocardioform bacteria can grow on similar kinds of media to mycobacteria, with incubation period ranging from 2 to 14 days. Growth of *Nocardia* appears as raised, folded, granular or powdery, pinkish-white to yellow-orange or light brown colonies with aerial mycelium around the edges (Isik *et al.*, 1999; Brown-Elliott *et al.*, 2006). Nocardial granulomas without the epithelioid cells in the earliest stages of development are confused easily with piscine mycobacteriosis.

Furthermore, while acid-fast organisms are present in the *Nocardia* lesion, they only show a positive reaction for *Nocardia* with Fite–Faraco acid-fast stain. Morphologically, *Nocardia* appear filamentous, branched and beaded and are 5–50 μm long, while mycobacteria are usually 1–3 μm in length (Wolke and Stroud, 1978).

More specific and sensitive methods have been applied recently for diagnosis, including antibody-based and DNA-based methods. Chen *et al.* (2000) described immunohistochemistry using polyclonal antibodies to detect *N. seriolae* in sea bass. However, a more specific probe such as monoclonal antibody has not been employed yet.

Polymerase chain reaction (PCR), employing primers to the 16S–23S rRNA internal transcribed spacer, was also developed to detect *N. seriolae* in yellowtail fish (Kono *et al.*, 2002). With the same nucleotide region, loop-mediated isothermal amplification (LAMP) was employed recently to detect *N. seriolae* infection in fish (Itano *et al.*, 2006a). As a sensitive and specific method, PCR could be useful for detection of the pathogen in both diseased fish and carriers.

Genome structure and transcription

Currently, there is no information on the *Nocardia* genome.

Pathogenesis and immunity

Nocardiosis is a systemic chronic granulomatous disease of fish caused by several species of *Nocardia*. Severe emaciation, inactivity and skin discoloration are the clinical signs of this disease. In advanced stages, cachexia, ascites, dermal ulceration, focal necrotic areas within skeletal muscle and pale areas in the swollen kidney, spleen, heart and liver may be observed (Kubota *et al.*, 1968). Small nodules are often observed in the internal organs (Fig. 11.5) and also on the gills of infected fish (Kusuda *et al.*, 1974).

Histopathological examination of infected organs should reveal the characteristic granulomas, each with a centre of necrotic material (Fig. 11.6), a peripheral cellular zone of numerous histiocytes, lymphocytes and a few multinucleated giant cells, all of which are partially circumscribed by a fibrous capsule. Colonies of acid-fast bacteria were present within and at the periphery of the granulomas (Labrie *et al.*, 2008). Hsu *et al.* (1987) observed numerous disseminated abscesses, encapsulated by granulation tissue, in the pleura and viscera of infected Formosa snakehead fish.

There have been attempts to develop vaccines against nocardiosis with a number of trials using yellowtail (*S. quinqueradiata*), an economically important fish species which is affected by the disease. Kusuda *et al.* (1989) demonstrated that yellowtails immunized with attenuated live *N. kampachi* showed an increase in the number of active lymphocytes and granulocytes within 72 h. Live vaccine, using *N. seriolae* related species including *N. soli*, *N. fluminea* and *N. uniformis*, revealed some levels of protective immune response to *N. seriolae* challenge in yellowtail (Itano *et al.*, 2006b).

Control, treatment and epizootiology

A number of antibiotics have been suggested for treating the disease and they include streptomycin and sulfasoxizole (Van Duijn, 1981; Frerichs and Roberts, 1989). Chen *et al.* (2000) suggested that a treatment with trimethoprim and sulfamethoxazole at 40 mg/l in feed with the addition of benzalkonium chloride at 2 mg/l in water decreased the mortality rates of the infected fish. However, given that these drugs are not totally effective, from an economic point of view it might be difficult to justify their use.

The development of nocardiosis in fish probably results from environmental stress and so the best method of disease control is by providing good husbandry in the culture system (Kusuda and Nakagawa, 1978). In aquarium fish, stressors such as overcrowding, elevated water temperature (above optimum)

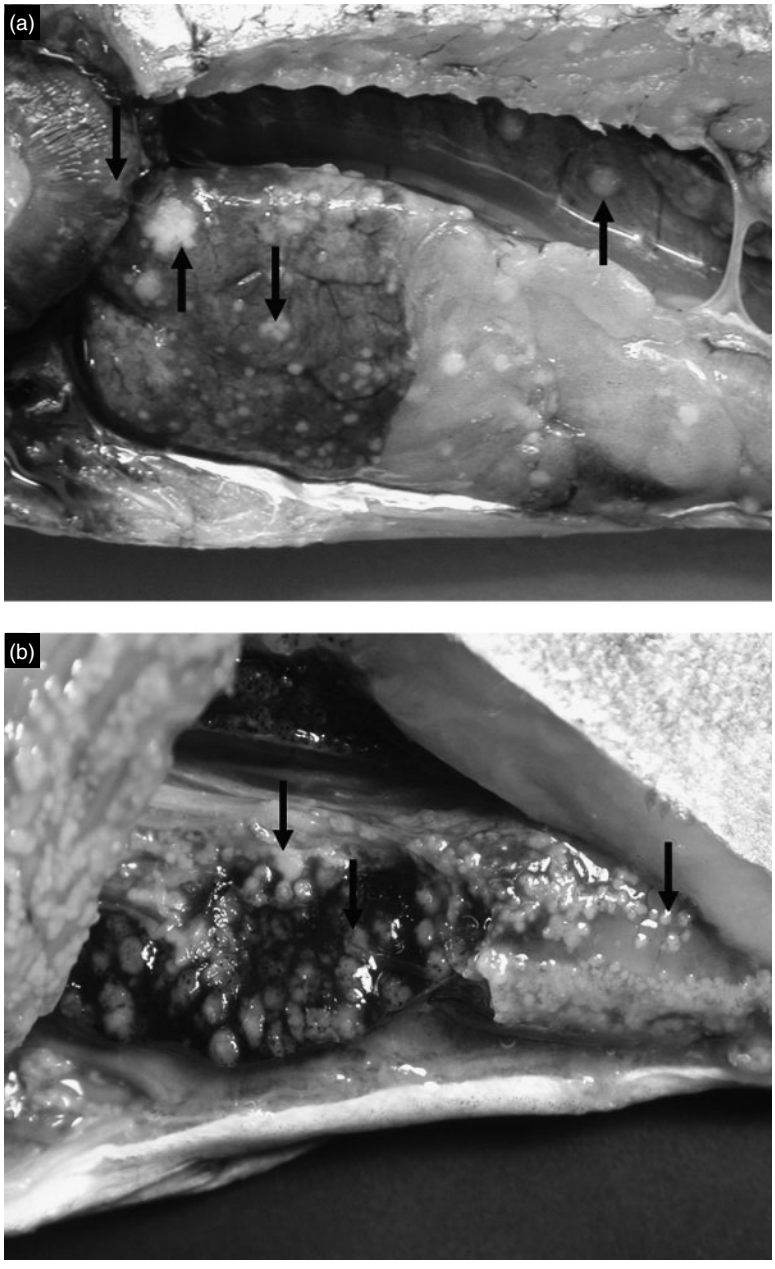


Fig. 11.5. Small nodules are common signs found in the internal organs and gills of the infected fish: (a) largemouth bass (*Micropterus salmoides*) infected by *Nocardia asteroides* showing numerous nodular structures in gill, liver and swim bladder (arrows); and (b) amberjack (*Seriola dumerili*) infected by *N. seriolae* showing numerous nodular lesions in spleen and mensentery (arrows). Courtesy of S.-C. Chen.

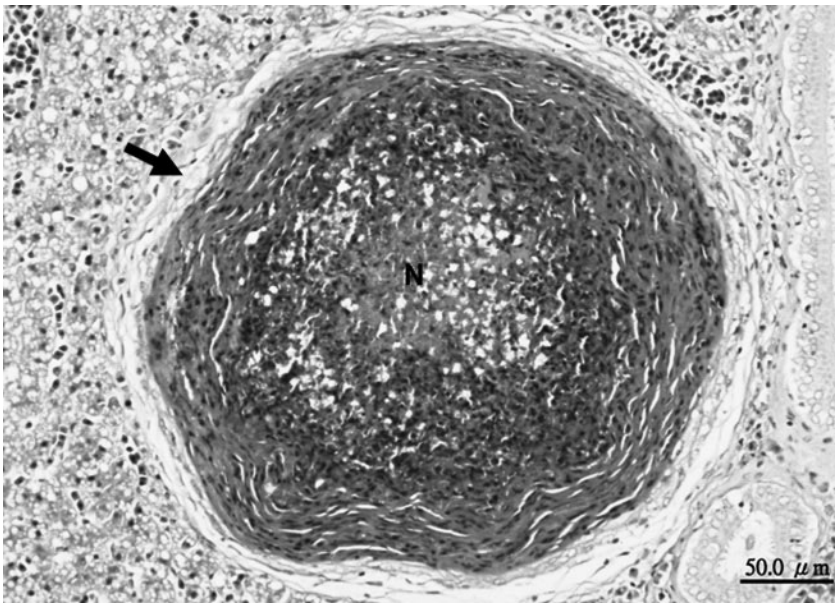


Fig. 11.6. Typical granuloma with necrotic centre (N) found in the liver of greater amberjack (*Seriola dumerili*) infected with *Nocardia seriolae* (H & E stain, ×200). Courtesy of S.-C. Chen.

or inadequate nutrients are predisposing causes of this disease (Conroy, 1964b; Ghittino, 1972).

Conclusions and recommendations for future studies

The taxonomy of bacteria in the genus *Nocardia* is not as well organized as in other groups (Austin and Austin, 1987), so there is a need for bacteriological taxonomists to modify the classification of nocardioform bacteria to help eliminate any confusion.

Vaccine development and molecular information regarding piscine nocardiosis are still very much in their infancy.

Acknowledgements

We would like to thank Dr Kim Thompson for her valuable comments and editorial advice on the genome section and also to thank Dr Philip N. Lewis for reviewing the manuscript. Special thanks are due to Prof. Shih-Chu Chen for providing the photographs for the nocardiosis section.

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12 Furunculosis and Other Aeromonad Diseases

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Introduction

Aeromonads have broad impacts on aquatic animal health. The motile species, *Aeromonas hydrophila*, has worldwide distribution, is among the most common bacteria in freshwater habitats and it frequently causes disease in cultured and feral fish (Austin and Austin, 2007). Reports in the early scientific literature provide circumstantial evidence of septicaemic infections caused by motile aeromonads in fish throughout Europe during the Middle Ages (Otte, 1963). Although the bacterial aetiology in these early reports was inconclusive, the pathology that was observed was similar to that documented for red leg disease in frogs, in which *A. hydrophila* was identified as the aetiological agent. Because many bacteria isolated from fish with haemorrhagic septicaemias were often misidentified, it has now been accepted that certain bacterial isolations ascribed to *Pseudomonas*, *Proteus*, *Bacillus*, *Aerobacter* and *Achromobacter* actually were *Aeromonas* (Kluyver and van Niel, 1936). Motile aeromonads are often referred to as a complex of disease organisms that are associated with bacterial haemorrhagic septicaemias and other ulcerative conditions in fish. Indeed, with the modern developments in bacterial systematics, the accuracy of early identifications may often seem questionable. Notwithstanding, motile aeromonads are ubiquitous in most freshwater environments and are common in the water column and in the upper layers of sediment (Hazen, 1979). They are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity and temperature (Hazen *et al.*, 1978a). Temperature optima may depend on the particular strain, but generally range from 25 to 35°C. Consequently, most epizootics in warmwater fish in the south-eastern USA are generally reported in the spring and early summer (Meyer, 1970). Outbreaks of aeromonad septicaemias in frogs and warmwater fish usually occur in the spring and coincide with an increase in water temperature. Resistance to disease is also lowered at this time because aquatic organisms are often anaemic and have a substantial decrease in serum proteins resulting from periods of dormancy and starvation that have occurred during the winter. Also, Huizinga *et al.* (1979) indicated that rising water temperatures increased metabolism, decreased overall condition and stressed the fish. These animals increased production of corticosteroids, which in turn increased their susceptibility to infection.

Similarly, epizootics of *A. hydrophila* and *A. jandaei* are frequently reported in

© CAB International 2011. *Fish Diseases and Disorders* Vol. 3: *Viral, Bacterial and Fungal Infections*, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

European eels (*Anguilla anguilla*) and most often occur in spring and summer when water temperatures range between 17 and 20°C (Esteve *et al.*, 1993). There has been some work indicating a pathogenic role for *A. allosaccharophila*, *A. caviae* and *A. sobria* in elvers, Atlantic salmon (*Salmo salar*)/rainbow trout (*Oncorhynchus mykiss*) and gizzard shad (*Dorosoma cepedianum*)/perch (*Perca fluviatilis*), respectively (Toranzo *et al.*, 1989; Martinez-Murcia *et al.*, 1992; Candan *et al.*, 1995; Ogara *et al.*, 1998; Wahli *et al.*, 2005). In the USA, motile aeromonads primarily cause disease in cultured warmwater fish: minnows, bait fish, carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*) and tilapia (*Oreochromis niloticus*) and their hybrids. The pathogen may also affect cool- and coldwater species, but is not necessarily restricted to freshwater environments. For example, in brackish waters, Rahim *et al.* (1985) isolated *A. hydrophila* from wounds of striped sea catfish (*Plotosus anguillaris*), barramundi (*Lates calcarifer*), spotted grouper (*Epinephelus megachir*), rohu (*Labeo rohita*) and blue tilapia (*Serotherodon nilotica*). Motile aeromonads have also been cultured from raw and processed products of marine fish, as well as from marine fishing grounds (Thampuran and Surendran, 1995). Although diseases associated with motile aeromonads are most severe in fish that are propagated under intensive culture, these bacteria also affect feral fish. It is also important to note that these organisms are often associated with the intestinal flora of apparently healthy fish (Trust *et al.*, 1974; Austin, 2006).

Motile aeromonads can also cause disease in warm-blooded vertebrates. In immunocompromised humans, *A. hydrophila* may cause septic arthritis, diarrhoea, corneal ulcers, skin and wound infections, meningitis and fulminating septicaemias (von Gravenitz and Mensch, 1968; Davis *et al.*, 1978). Clinical isolates of *A. hydrophila* have been obtained from retail foods (fish, seafood, raw milk, poultry and red meats) and all isolates had biotypes identical to those of enterotoxin-positive strains (Palumbo *et al.*, 1989). The ability of these bacteria to grow competitively at 5°C may indicate their potential as a public health hazard. However, most *A. hydrophila* were reduced to non-detectable levels on catfish fillets cooked to 70°C (Huang *et al.*, 1993).

In contrast to the diversity that exists among the complex of motile aeromonads, the non-motile *A. salmonicida* is a more homogeneous species commonly associated with systemic and ulcerative conditions in fish. Emmerich and Weibel (1890) first described *A. salmonicida* (syn. *Bacillus salmonicida*, *Bacterium salmonicida*, *B. trutta*) from diseased trout in German hatcheries, and it was believed initially that the organism was a pathogen exclusive to intensively cultured salmonid fish. Current knowledge, however, indicates that relatively few cultured or feral fish in freshwater, brackish or even marine environments are immune to this pathogen. All species of salmon, trout, charr and grayling are susceptible to infection. Furthermore, the number of non-salmonid hosts has multiplied steadily commensurate with increased epidemiological investigations. Present indications suggest that there are at least five subspecies of *A. salmonicida*: subsp. *salmonicida*, subsp. *achromogenes*, subsp. *masoucida*, subsp. *smithia* and subsp. *pectinolytica* (Pavan *et al.*, 2000; Martin-Carnahan and Joseph, 2005). However, the validity of the subspecies scheme in view of the comparatively large number of atypical isolates is questionable. Strains of *A. salmonicida* subsp. *salmonicida* most commonly induce typical furunculosis and cause severe septicaemia with resultant mortality, especially in coldwater fish. The other subspecies, except *pectinolytica*, which has not been associated with fish pathology (Pavan *et al.*, 2000), produce atypical forms of disease that are often characterized by dermal ulcerations and external pathology with or without subsequent septicaemia. The number, diversity and distribution of fish species that are susceptible to *A. salmonicida* enhance this bacterium's distribution worldwide. However, both typical and atypical subspecies may not be common to all geographic areas. For example, *A. salmonicida* was not reported in

New Zealand or Australia until 1974, when atypical variants of *A. salmonicida* were introduced to Australia by importation of diseased goldfish (Trust *et al.*, 1980a; Humphrey and Ashburner, 1993). Since that introduction, atypical isolates of *A. salmonicida* have become endemic, even in feral populations of goldfish (Whittington *et al.*, 1987), but *A. salmonicida* subsp. *salmonicida* has not been reported in Australian fish. Similarly, only *A. salmonicida* subsp. *salmonicida* has been reported from salmonids in Nova Scotia, Canada (Hammell, 1995). It may be argued, however, that such distinctions are somewhat controversial because atypical isolates of *A. salmonicida* from goldfish (*Carassius auratus*) can be virulent in Atlantic salmon (*Salmo salar*) and induce pathology indicative of classical furunculosis (Carson and Handlinger, 1988). Diversity within the species definition of *A. salmonicida* was highlighted by Smith (1963), who studied six non-pigmented cultures, which were grouped together as a result of numerical taxonomy and related at the 75.6% similarity level to typical pigment-producing isolates. She proposed a separate species – *achromogenes*. A second non-pigmented group was also recognized by Kimura (1969) as *masoucida*. Smith (1963) proposed that the fish pathogens be transferred to a new genus, *Necromonas*, with two species *N. achromogenes* and *N. salmonicida*, but the proposal was not accepted.

Classification

Kluyver and van Niel (1936) transferred many organisms (genera *Bacillus*, *Pseudomonas*, *Proteus* and *Aerobacter*) that were associated with haemorrhagic septicaemias in fish into the new genus *Aeromonas*. These aeromonads were short, Gram-negative, motile bacilli with single flagella that fermented glucose with or without the production of gas. Snieszko (1957) later divided the genus into three species: *A. hydrophila*, *A. punctata* and *A. liquefaciens*. In Snieszko's scheme, *A. liquefaciens* contained most of the bacterial strains that were pathogenic for fish. Schubert (1967) confirmed that there was enough biochemical similarity to establish the genus *Aeromonas*, but invalidated species-specific distinctions. Later, Popoff and Vernon (1976) demonstrated that the motile aeromonads could be classified into two distinct species: *A. hydrophila* (composed of the organisms described previously as *A. punctata* and *A. liquefaciens*) and a new species designated as *A. sobria*. Biochemically, *A. hydrophila* hydrolysed aesculin and fermented salicin and arabinose, whereas *A. sobria* did not (Lallier *et al.*, 1981).

Motile aeromonads may be pleomorphic, but generally produce circular, smooth, raised colonies on agar. The bacteria are short ($0.5 \times 1.0 \mu$m), and Gram-negative. Phenotypically, motile aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to the vibriostatic agent 0/129 (2,4-diamino, 6,7-di-isopropyl pteridine). In addition, the bacteria produce 2,3-butanediol and reduce nitrate to nitrite. Hsu *et al.* (1985) noted that all the isolates of the motile aeromonads they studied produced acid from fructose, galactose, maltose, mannitol, trehalose, dextrin and glycogen; and 99.4% of the strains produced acid from glucose, 98.8% from mannose and 98.2% from glycerol. Acid production from other carbohydrates (arabinose, salicin, cellobiose, sucrose and lactose) varied. Shotts *et al.* (1985) also found that motile aeromonads hydrolysed albumin, casein and fibrinogen; most strains also digested gelatin (99.9%), haemoglobin (94.3%) and elastin (73.2%), but none of their strains hydrolysed collagen. Further phenotypic differentiation of the seven most commonly isolated motile aeromonads obtained from clinical isolates (Table 12.1) were accomplished using the criteria of Carnahan *et al.* (1991), as modified by Joseph and Carnahan (1994). Of these organisms, Austin *et al.* (1989) showed that *A. hydrophila*, *A. sobria* and *A. caviae* were the most prevalent clinical isolates associated with fish disease. Noterdaeme *et al.* (1996) found that *A. hydrophila*, *A. caviae* and *A. veronii* biotype *sobria* formed discrete clusters phenotypically. They noted the presence of two biotypes among their isolates of *A. caviae*

Table 12.1. Differentiation of some common motile aeromonads isolated from clinical specimens as described by Carnahan *et al.* (1991, as modified by Joseph and Carnahan, 1994). All bacterial isolates are short Gram-negative, oxidase-positive bacilli that ferment glucose and are resistant to the vibrostatic agent, 0129.

Characteristic[a]	*A. hydrophila*	*A. veronii* bv. *sobria*	*A. veronii* bv. *veronii*	*A. caviae*	*A. schubertii*	*A. jandaei*	*A. trota*
Aesculin hydrolysis	+	–	+	+	–	–	–
Voges–Proskauer reaction	+	+	+	–	V	+	–
Pyrazinamidase activity	+	–	–	+	–	–	–
CAMP-like factor (aerobic only)	+	+	+	–	–	V	–
Arabinose fermentation	V	–	–	+	–	–	–
Mannitol fermentation	+	+	+	+	–	+	+
Sucrose fermentation	+	+	+	+	–	–	–
Ampicillin susceptibility	R	R	R	R	R	R	S
Carbenicillin susceptibility	R	R	R	R	R	R	S
Cephalothin susceptibility	R	S	S	R	S	R	R
Colistin susceptibility[b]	V	S	S	S	S	R	S
Lysine decarboxylase	+	+	+	–	+	+	+
Ornithine decarboxylase	–	–	+	–	–	–	–
Arbutin hydrolysis	+	–	+	+	–	–	V
Indole production	+	+		+	–	+	+
H_2S production	+	+	+	–	–	+	+
Gas from glucose	+	+	+	–	–	+	+
Haemolysis (TSA with 5% sheep erythrocytes)	+	+	+	V	+	+	V

[a]+, positive for >70% of isolates; –, negative, i.e. positive for <30% of isolates; V, variable; R, resistant; S, susceptible.
[b]MIC (single dilution), 4 μg/ml.

that were differentiated by fermentation of mannose. Another species, *A. schubertii* (type strain ATCC 43700) formed a single member cluster, whereas *A. media* (type strain ATCC 33907) and *A. eucrenophila* (type strain CDC 0859-83) were not well delineated phenotypically. Results obtained by phenotypic characterization and numerical taxonomy have not always agreed with DNA hybridization. For example, *A. veronii* biotype *veronii* (type strain ATCC 35624) was close to *A. hydrophila* phenotypically, but hybridization results associated it more closely with *A. sobria* (Noterdaeme *et al.*, 1996). Kaznowski (1998) divided the aeromonads into seven hybridization groups. Strains from one genomic species were sometimes categorized in different phenotypic clusters and, in some cases, a discrete phenotypic cluster could contain multiple

hybridization groups (Kaznowski, 1997). Subsequently, Valera and Esteve (2002) concluded that phenotypic characterization was necessary for identification of aeromonad species.

In the ninth edition of *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994) and the subsequent edition (Martin-Carnahan and Joseph, 2005), four subspecies of *A. salmonicida* were recognized; namely, subsp. *salmonicida*, subsp. *masoucida*, subsp. *achromogenes* and subsp. *smithia* (Table 12.2). Although phenotypic (Griffin, 1954; Popoff, 1969; Dalsgaard *et al.*, 1994; Hanninen *et al.*, 1995) and genotypic (Belland and Trust, 1988; Boyd *et al.*, 1994; Nielsen *et al.*, 1994; Umelo and Trust, 1998; Garcia *et al.*, 2000; O'Hici *et al.*, 2000) characterizations have indicated that *A. salmonicida* subsp. *salmonicida* forms a homogeneous clonal group, some variation exists among the three other subspecies, referred to collectively as 'atypical' strains (Powell *et al.*, 1998; Høie *et al.*, 1999; Yamada *et al.*, 2000).

Umelo and Trust (1998) found that the circular chromosome of *A. salmonicida* was approximately 4658+/−30 kb. Their analysis of I-CeuI genomic digestion fingerprints confirmed the homogeneity of subsp. *salmonicida* typical strains, but also revealed extensive diversity among atypical strains. Nilsson *et al.* (2006) also found that the *tapA* gene of atypical *A. salmonicida* was interrupted by an insertion sequence, referred to as *ISAsa4*. Except for the presence of the insertion sequence (*ISAsa4*) among atypical isolates, their *tapA* sequence was virtually identical to typical isolates. Atypical isolates carried many copies of the insertion sequence, which suggested that *ISAsa4* might account for diversity among genetically heterogeneous atypical *A. salmonicida*. Sorum *et al.* (1993) reported that plasmid profiles from *A. salmonicida* subsp. *salmonicida* were some-

Table 12.2. Phenotypic differentiation of *Aeromonas salmonicida* as described in *Bergey's Manual of Determinative Bacteriology*, 9th edition (Holt *et al.*, 1994) and updated in the subsequent edition (Martin-Carnahan and Joseph, 2005).

	Aeromonas salmonicida subsp.			
Characteristic	*salmonicida*	*achromogenes*	*masoucida*	*smithia*
Indole production	–	+	+	–
Methyl red	+	+	+	–
Voges–Proskauer	–	–	+	–
H_2S production	–	–	+	+
Lysine decarboxylase	d	d	d	–
Arginine dihydrolase	+	+	+	[–]
D-Glucose acid	+	+	+	[+]
D-Glucose gas	+	–	+	[+]
L-Arabinose acid	+	–	+	
D-Galactose acid	+	+	+	–
Glycerol acid	d	d	d	[–]
Maltose acid	+	+	+	–
D-Mannitol acid	+	–	+	–
Sucrose acid	–	+	+	d
Trehalose acid	+	+	+	–
Aesculin hydrolysis	+	–	+	–
Lipase (corn oil)	+	+	+	–
ONPG	d	d	d	+
Brown pigment	+	–	–	–

+, 90% or more strains are positive; –, 90% or more strains are negative; [–], 0–10% are positive; d, 11–75% of strains are positive; [+], 76–89% of strains are positive.

what homogeneous, whereas those from atypical isolates showed some diversity that could possibly be used as a measure to assist epidemiological typing (Sorum *et al.*, 2000). Ribotyping of chromosomal DNA digested with SmaI, Bg/I, PstI and CLI has also proven to be somewhat successful in distinguishing epidemiological differences among Finnish isolates of *A. salmonicida* subsp. *salmonicida* from those obtained in Denmark, Sweden, Norway and Canada (Hanninen *et al.*, 1995).

A number of other strains of *A. salmonicida* have been identified that do not conform to the current classification scheme based on four subspecies (Austin *et al.*, 1998). Austin *et al.* (1989) actually divided *A. salmonicida* into five phena. In those studies, subspecies *masoucida*, *salmonicida*, *achromogenes* and *smithia* were grouped within phena 10, 11, 12 and 14, respectively. Phenon 13 contained the additional group of atypical isolates that did not group within any of the recognized subspecies. Isolates from this phenon have been obtained from both salmonid and non-salmonid sources, can induce ulcers and septicaemia in rainbow trout and goldfish and produce brown pigment. Unlike all previously reported isolates of *A. salmonicida*, most (87%) of the isolates in phenon 13 can grow at 37°C (Austin, 1993). Based on additional phenotypic and DNA hybridization differences, a new subspecies, '*pectinolytica*' has been proposed for isolates of *A. salmonicida* obtained from polluted waters of the Mantanza River near Buenos Aires, Brazil (Pavan *et al.*, 2000). These isolates fermented sorbitol and degraded polypectate. Analysis of 16S rDNA sequences indicated that these isolates formed a tight genomic group that was related most closely to *A. salmonicida* subsp. *salmonicida*, *masoucida* and *achromogenes*. In addition to these differences, Martinez-Murcia *et al.* (2006) determined that *A. salmonicida* and *A. bestiarum* could not be differentiated by their 16S rDNA sequences. These investigators determined that there was a 75.6% DNA–DNA hybridization similarity between the type strains of both species. A phylogenetic divergence was noted between the two species in their respective *gyrB* and *rpoD* gene sequences.

Pathogenicity and Clinical Signs

Motile aeromonads cause diverse pathological conditions that include acute, chronic and covert infections. Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiological condition of the host and the degree of genetic resistance inherent within specific populations. Motile aeromonads differ interspecifically and intraspecifically in their relative pathogenicity or their ability to cause disease. Under controlled laboratory conditions, De Figueredo and Plumb (1977) found that strains of motile aeromonads isolated from diseased fish were more virulent to channel catfish than those from pond water. Lallier *et al.* (1981) performed studies on rainbow trout (*Oncorhynchus mykiss*, formerly *S. gairdneri*) to compare the relative virulence of *A. hydrophila* and *A. sobria*, as described by Popoff and Véron (1976). Their results indicated that strains of *A. hydrophila* isolated from either healthy or diseased fish were more virulent than strains of *A. sobria.* Additionally, *A. sobria* was not isolated from fish with clinical signs of motile aeromonad septicaemia (Boulanger *et al.*, 1977). Paniagua *et al.* (1990), for example, collected aeromonad isolates along the River Porma, Leon Province (Spain) and found that their isolates grouped within three species; *A. hydrophila* (n=74 strains), *A. sobria* (n=11 strains) and *A. caviae* (n=12 strains). The authors additionally observed that 72% of *A. hydrophila* isolates and 63% of *A. sobria* isolates were virulent to fish by intramuscular challenge, but all of the strains of *A. caviae* were avirulent. Pathological conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, haemorrhagic septicaemia, red sore disease, red rot disease and scale protrusion disease.

In the acute form of a motile aeromonad disease, a fatal septicaemia may occur so rapidly that fish die before they have time to develop anything but a few gross signs of disease. When clinical signs of infection are present, affected fish may show exophthalmia,

reddening of the skin and an accumulation of fluid in the scale pockets (Faktorovich, 1969). The abdomen may become distended as a result of an oedema and the scales may bristle out from the skin to give a 'washboard' appearance. The gills may haemorrhage and ulcers may develop on the dermis. Ogara *et al.* (1998) noted severe eye pathology and heavy mortality among yearling and older rainbow trout accompanying a severe outbreak of motile aeromonad septicaemia. The condition at first affected one eye, progressed into the second, after which the orbits ruptured, causing blindness and death. Similarly, Yambot and Inglis (1994) described an acute mortality among Nile tilapia in which the most apparent clinical signs included opaqueness in one or both eyes, accompanied by exophthalmia and eventual bursting of the orbit. Motile aeromonads were isolated from the eyes, liver and kidneys of affected fish. Histopathologically, fish may exhibit epithelial hyperplasia in the foregut, leptomeningeal congestion in the brain, as well as a thrombosis and inflammation in the perisclerotic region and corneal epithelium of the eye (Fuentes and Perez, 1998). There may also be a severe branchitis, as indicated by leucocytic infiltration and dilation of the central venous sinus (Grizzle and Kiryu, 1993). These authors also noted that catfish with septicaemic or latent infections had enlarged nuclei in the branchial epithelium and that there was a significant correlation between the presence of these gill lesions and the severity of hepatic and pancreatic lesions. Rodriguez *et al.* (1993a,b) further noted that there was an increase in bacterial acetylcholinesterase activity in the brain tissue of moribund fish.

Systemic infections are characterized by diffuse necrosis in several internal organs and the presence of melanin-containing macrophages in the blood (Ventura and Grizzle, 1988). Internally, the liver and kidneys are target organs. The liver may become pale, or have a greenish coloration, while the kidney may become swollen and friable. These organs are apparently attacked by bacterial toxins and lose structural integrity (Huizinga *et al.*, 1979). Even when tissue damage in the liver and kidneys is extensive, the heart and spleen are not necessarily damaged. However, splenic ellipsoids are often centres of intense phagocytic activity by macrophages. Bach *et al.* (1978) observed pathological changes in the spleens of fish injected with virulent *A. hydrophila*, whereas fish infected orally showed little or no splenic involvement. Bacteria were present within the reticular sheaths of the ellipsoids, where intense phagocytic activity by macrophages occurred. Phagocytized bacteria divided intracellularly and extracellularly and destroyed the endothelial and reticular cells of the ellipsoids. The lower intestine and vent, which sometimes protrude from the body, are often swollen, inflamed and haemorrhagic. Additionally, the intestine is devoid of food and may be filled with a yellow mucus-like material.

Chronic motile aeromonad infections manifest themselves primarily as ulcerous forms of disease, in which dermal lesions with focal haemorrhages and inflammation are apparent. Both the dermis and epidermis are eroded and the underlying musculature becomes severely necrotic (Huizinga *et al.*, 1979). Inflammatory cells are usually lacking in the necrotic musculature, whereas the adjacent epidermis undergoes a hyperplasia that results in a raised margin. At this stage, the infection has usually become systemic and petechial haemorrhages may occur throughout the peritoneum and musculature. Fish with only cutaneous infections may have several types of concealed lesions, including increased amounts of lipofuscin and haemosiderin in the liver and spleen; however, most visceral organs were not necrotic (Ventura and Grizzle, 1988). Hepatic necrosis has been observed in channel catfish with systemic, cutaneous and latent infections caused by *A. hydrophila*, whereas hepatic steatosis was only evident in systemic infections (Grizzle and Kiryu, 1993). In these same studies, pancreatic atrophy and necrosis were also evident in systemically diseased fish. Infiltration of leucocytes in necrotic areas of the liver and intrahepatic exocrine pancreas of infected fish was not observed, nor was there any correlation between infection and hepatic haemorrhage or haemosiderosis.

Although spongiosis of gill epithelium was apparent in systemically infected, cutaneously infected and latently infected fish, severe bronchitis was most evident in systemically infected fish. The systemically infected fish also had enlarged nuclei in branchial epithelia. In eels (*A. japonica*), *A. hydrophila* characteristically induced necrosis of muscle bundles, septic haemorrhages, lesions in the spleen, fatty livers, atrophy of renal haematopoietic tissue and necrosis in nephrons (Chien and Chieh, 1994).

A. hydrophila generally was considered to be a secondary invader in red sore disease, in which the primary aetiological agent was believed to be the protozoan ciliate *Epistylis* (Rogers, 1971). Hazen *et al.* (1978b) re-examined the aetiology of red sore disease and found that *A. hydrophila* was present in 96% of the initial lesions on fish, whereas *Epistylis* was present in only 35% of the lesions. Furthermore, electron microscopy showed that *Epistylis* lacked structures that produced lytic enzymes and, therefore, could not initiate the development of lesions. This study strongly suggested that *A. hydrophila* was the primary aetiological agent of red sore disease and that *Epistylis* was a secondary pathogen that rapidly colonized the dermal lesions initiated by bacterial proteolytic enzymes. In frogs and other amphibians, *A. hydrophila* infections cause distension of capillaries on the ventral surface of the legs and abdomen, producing the red coloration from which the disease is named – red leg.

Some work has suggested a role for virulence plasmids in *A. hydrophila*. In one study, a pathogenic strain (coined VB21) that caused an ulcerative disease in Indian catfish (*Clarias batrachus*) had a 21 kb plasmid. Curing of this plasmid led to antibiotic sensitivity and alterations to structural characteristics of the host cell, including surface structure, and a change in virulence (Majumdar *et al.*, 2007a). Subsequent transformation of the plasmid back into the cured strain enhanced virulence (Majumdar *et al.*, 2007a).

A. salmonicida subsp. *salmonicida* causes severe septicaemia and acute mortality in susceptible salmonids. The mode of infection, nature of pathology and the degree of mortality are interrelated with the quality of environmental conditions, and by the age and innate resistance of the host. Peracute infections occur most often in fingerling fish, which may darken in colour and die without clinical signs of disease. Only a slight exophthalmia may be evident. Acute infections often occur in juvenile and adult fish, which darken in colour and haemorrhage at the base of fins and oral cavity. Internal haemorrhages may be evident in the abdominal walls, viscera and heart of affected fish. The spleen is enlarged and the liver can have subcapsular haemorrhages, or focal necrosis of parenchymatous tissue. Affected fish may display erratic swimming behaviour, become sluggish and stop feeding. Consequently, the stomach and intestine are usually devoid of food and the lumen may contain sloughed epithelial cells, mucus and blood. The reproductive organs are commonly haemorrhaged and the intestine is often severely congested (Scott, 1968). The chronic form of furunculosis usually occurs in older fish that have become more refractive to the disease or among species that have greater innate resistance to infection by *A. salmonicida*. One or more furuncle-like lesions may be present on the dermis and ulcers may extend deep into the musculature. Internally, chronically infected salmonids show a general visceral congestion and peritonitis. Haemorrhages may occur over the pyloric area and liver, and kidneys are soft or friable (McCarthy and Roberts, 1980).

The development of the characteristic 'furuncle-like' lesion is not a consistent finding, but is associated most often with chronic infections. When these lesions are present, they consist of tissue fluid exudate, necrotic tissue and some macrophages. Thus, the furunculosis lesion differs from the furuncle in homeothermic vertebrates, which is characterized by a necrotic mass of polymorphonuclear leucocytes. Degeneration of myofibrils, fragmentation of muscle fibres and haemorrhage of the entire muscular tissue are evident within the swelling lesion and lead to a colliquative necrosis of the musculature in the most serious lesions

(Sakai, 1978). Bacteria may also colonize the gill epithelium on or between the secondary lamellae (Bruno, 1986), where they may be enclosed within a membrane that is continuous with the basement membrane of the lamellar epithelium (McArdle *et al.*, 1986). Bacterial embolisms may develop in gill lamellae, causing a further proliferation of branchial epithelial cells and a subsequent fusion of gill lamellae that impairs circulation (Miyazaki and Kubota, 1975a).

In addition to typical furunculosis caused by *A. salmonicida* subsp. *salmonicida*, a number of other diverse pathological conditions may be caused by other 'atypical' subspecies. Fish (1934) described an ulcer disease in trout in salmonids from a hatchery in Cortland, New York, which he believed was distinct from the clinical picture observed in typical salmonid furunculosis. He also noted that a similar pathology had been reported previously in trout at two other New York hatcheries on Long Island (Calkins, 1899) and Cold Spring Harbor (Marsh, 1905). In each of these cases, the disease manifested itself as an external bacterial infection without involvement of the blood, liver, spleen and kidneys, but the posterior intestine was often congested. Lesions were observed infrequently in the kidneys or livers of affected fish and, if present, they were consistently sterile by culture on nutrient agar. Snieszko (1952) implied that an internal pathology similar to that described for typical salmonid furunculosis might have developed in the most acute cases. Because specific pathogen-free fish were not readily available, it was difficult to determine if the internal pathology alluded to by Snieszko was actually a clinical indication of Ulcer Disease or the expression of an underlying *A. salmonicida* subsp. *salmonicida* infection. The external clinical pathology that was observed in cases of Ulcer Disease, however, was quite dissimilar to typical salmonid furunculosis. Patches of the epidermis initially thickened and then enlarged to form white epidermal tufts (0.5–1.0 mm in diameter). The development of these tufts progressed until the underlying dermis was eroded and a finely delineated dark red or grey ulcer became apparent. Bacteria multiplied within the necrotic debris of the exposed subepithelial connective tissue but did not necrotize or penetrate deeply into the musculature. The lesions, therefore, remained shallow and developed from the outside in. By contrast, the furuncles of typical salmonid furunculosis developed from the inside out and were evident deep into the musculature. Other ulcers were also present on the fins, jaw, or in the oral cavity, where the soft tissue covering the roof of the mouth was eroded (Wolf, 1938). Snieszko and Friddle (1948) originally isolated and Snieszko *et al.* (1950) then described *Haemophilus piscium* as the aetiologic agent of Trout Ulcer Disease, although this organism was never proposed formally and lacked standing taxonomically. Paterson *et al.* (1980) later suggested that this bacterium was actually an atypical, achromogenic strain of *A. salmonicida* by showing that DNA hybridization results for *H. piscium* were similar to those for *A. salmonicida*, but beyond acceptable limits for the genus *Haemophilus*. These workers further showed that strains of *H. piscium* were: (i) sensitive to *A. salmonicida* bacteriophage; (ii) serologically identical to *A. salmonicida*; and (iii) phenotypically analogous to other achromogenic strains of *A. salmonicida*. Unfortunately, the original isolates described by Snieszko and Friddle (1948) and Snieszko *et al.* (1950) were not available for comparison. Thornton *et al.* (1999) also showed the SSU rRNA gene sequence identity between *H. piscium* and *A. salmonicida* subsp. *salmonicida* was 99.6%. This information strongly suggested that the pathology observed in Trout Ulcer Disease was caused by an atypical, achromogenic strain of *A. salmonicida*.

Goldfish Ulcer Disease was another of the atypical conditions caused by this bacterium. The condition was first described by Mawdesley-Thomas (1969), who termed the condition furunculosis of goldfish because *A. salmonicida* was isolated from skin lesions. However, the organism in those studies was actually a typical biotype of the bacterium, *A. salmonicida* subsp.

salmonicida, and, therefore, the clinical pathology described by Mawdsley-Thomas was quite different from that which was observed in the majority of reports concerning Goldfish Ulcer Disease. True Goldfish Ulcer Disease, however, was actually introduced into goldfish farms in the USA during the 1970s. Although other investigators attributed the aetiology of this disease to a fungus (oomycete), a yellow pigmented bacterium (*Flavobacterium columnare*), or a protozoan (*E. longicorpia*), Elliott and Shotts (1980a,b) established that the actual causal organism was another atypical, achromogenic variant of A. *salmonicida*. Although the role of ectoparasites in creating a portal of entry for *A. salmonicida* remained unclear, *A. salmonicida* preferentially adhered to mucus and dead or damaged cells in the dermis. Consequently, early Goldfish Ulcer Disease infections appeared as white proliferations on the epithelium of infected fish. These areas developed peripheral haemorrhages beneath the scales. As the lesion progressed, scales were sloughed, the dermis became necrotic and there was significant degeneration of the musculature. There was also a marked leucocyte infiltration at the site of trauma within 24–48 h. Unlike typical salmonid furunculosis, the infiltration persisted throughout a 21-day observation period and consisted of acidophilic, heterophylic and basophilic granulocytes, lymphocytes and macrophages. There was no evidence, however, that *A. salmonicida* was phagocytized by the leucocytes. Septicaemia caused by *A. salmonicida* was most likely to be found if fish had well-developed dermal lesions. The causative bacterium was isolated most readily just below the dermis at the peripheral margin of the lesion. *A. salmonicida* was recovered most reliably from early-stage skin lesions, whereas secondary infections established by opportunistic strains (e.g. *A. sobria* and *A. hydrophila*) became prominent within late-stage ulcers (Dror *et al.*, 2006).

Another atypical *A. salmonicida* infection, commonly referred to as Carp Erythrodermatitis, was described as a subacute to chronic skin disease of carp (Pol *et al.*, 1980). This disease was associated historically with Carp Dropsy Syndrome until Fijan (1972) showed that Carp Dropsy actually involved two infections: Spring Viraemia of Carp caused by *Rhabdovirus carpio* and Carp Erythrodermatitis. Consequently, the aetiologic agent of Carp Erythrodermatitis was isolated and described as an atypical, achromogenic variant of *A. salmonicida* (Bootsma *et al.*, 1977; Bootsma and Blommaert, 1978). Although *A. salmonicida* is now recognized to cause Carp Erythrodermatitis, it is important to note that other species of bacteria may also induce external lesions in carp that are macroscopically similar to those that are caused by *A. salmonicida* (Schultz, 1980). Clinical indications of Carp Erythrodermatitis become evident as small inflamed haemorrhagic areas on the skin that develop into white erosions surrounded by a narrow red zone and darkened pigmentation. Extensive haemorrhage in the dermis is followed by necrosis of the epidermis, with strong infiltration by polymorphonuclear leucocytes. Oedema in the dermis displaces 'knots' in the epidermis, while haemorrhage occurs around the veins to induce a persistent inflammatory infiltration that extends into the stratum compactum, subdermis and underlying musculature. Irregular haemorrhagic ulcers proliferate in the central necrotic areas (Gayer *et al.*, 1980).

Transmission and Bacterial Ecology

Contaminated pond water, diseased fish and diseased frogs, as well as convalescent frogs and fish, may be reservoirs of infection. Certain algae (Kawakami and Hashimoto, 1978) and other protozoa that are grazed upon by fish can also harbour motile aeromonads (Chang and Huang, 1981). In the latter study, *Tetrahymena pyriformis* was shown experimentally to graze on populations of *A. hydrophila*. Hazen (1979) found high densities of *A. hydrophila* in mats of decomposing *Mypiophyllum spiaatum* and within the intestines of largemouth bass, turtles, alligators and snails. Kawakami

and Hashimoto (1978) found greater quantities of *A. hydrophila* (10^3 cells) in algae fed upon by ayu (*Plecoglossus altivelis*) than were present in the water itself, where the density of *A. hydrophila* was generally less than 10 cells/ml. Osborne *et al.* (1989) found high densities of motile aeromonads within the environment during midsummer when sedimentary chlorophyll *a* and water temperature were highest. This also correlated temporally with the highest prevalence of dermal ulcerated mullet (*Mugil cephalus*) that also harboured heavy concentrations of the bacteria within their stomachs and on their skin.

Because these bacteria compose part of the normal intestinal microflora of healthy fish (Trust *et al.*, 1974), the presence of the bacteria, by itself, is not indicative of disease. Consequently, the stresses associated with intensive aquaculture are often considered to enhance the severity of epizootics caused by the bacteria. As one such example, Eisa *et al.* (1994) showed that the prevalence of motile aeromonad septicaemia was more severe (10.0%) among cultured versus wild tilapia (2.5%). They also showed that the prevalence of septicaemia was 18.8% in cultured Karmout catfish (*C. lazera*), but only 6.3% in wild catfish. Ventura and Grizzle (1987) produced systemic infections more readily in channel catfish by abrading their skin before the fish were exposed to the bacterium. These researchers further showed that *A. hydrophila* could infect internal organs via the digestive tract or through uninjured skin when the fish were crowded (13.1 g of fish/l) and held at higher temperatures (≥24°C). Infections were not produced when catfish were held at a lower density (5.2 g of fish/l) and at cooler temperatures (≤18°C). In another interesting study, Peters *et al.* (1988) subjected subordinate rainbow trout to social stresses of cohabitation with dominant cohorts and then exposed these fish to *A. hydrophila*. By comparison to their dominant counterparts, the subordinate trout showed physical evidence of stress based on elevated plasma glucose concentrations and increased leucocyte volumes. Following exposure to the pathogen, the bacterium was also recovered from more internal organs and with greater prevalence among the subordinate fish than from their dominant cohorts. In feral fish, other factors may be important stressors that trigger motile aeromonad septicaemias. Toranzo *et al.* (1989), for example, described the occurrence of such an epizootic associated with spawning stress in gizzard shad (*D. cepedianum*) in the Potomac River, Maryland, USA. Ortega *et al.* (1996) determined that aeromonads were more likely to cause disease in fish if there was less than 7.06 mg/l of oxygen and more than 0.05 mg/l of ammonia in their culture water. Parasite infestations, such as those caused by *Ichthyophthirius multifiliis*, can also stress fish and cause mechanical trauma in terms of minute dermal ulcerations that facilitate invasion (Liu and Lu, 2004). *A. hydrophila* can enter a non-culturable but viable state (NCBV) after 50 days in 0.35% NaCl (pH 7.5) at 25°C (Rahman *et al.*, 2001). Although cells could not be cultured, 10^4 cells/ml were detected by microscopy after staining with fluorescein diacetate and ethidium bromide (Rahman *et al.*, 2001). These cells, however, were not pathogenic for goldfish. Therefore, the relevance of these cells to fish health is unclear.

By contrast to the ubiquitous motile, facultatively pathogenic aeromonads, *A. salmonicida* is regarded as an obligate pathogen. Fish that harbour latent infections of *A. salmonicida* can transmit the pathogen horizontally either by physical contact or by shedding the bacterium into the water column. Such transmission has been demonstrated in both fresh water (Horne, 1928) and marine environments (Scott, 1968; Nomura, 1993). Among Atlantic salmon, furunculosis epizootics are particularly troublesome at the beginning of marine culture if undetected *A. salmonicida* carrier smolts are transported into net pens (Hammell, 1995). Under such conditions, transport often facilitates contagion and the physiological stress encountered with osmoregulation may enhance disease (Ezura *et al.*, 1984). Contagion within natural waterways may be further enhanced by the escape of infected individuals from culture facilities or sea farms and the subsequent

movements of these infected fish within the feral environment (Johnsen and Jensen, 1994). Tank studies have shown that the bacterium is released into the surrounding water column once fish begin to die (Enger *et al.*, 1992). Under experimental conditions, furunculosis-dependent mortality rates in Chinook salmon (*O. tshawytscha*) decreased with decreasing host densities, but low mortality did not necessarily equate with a low rate of infection (Ogut *et al.*, 2005). Glenn and Taylor (2006) also found that infected steelhead (*O. mykiss*) held in high densities (3.99 fish/l) were 1.6 times more likely to die than those that were held at lower densities (1.59 fish/l). By extrapolation to the natural environment, therefore, large aggregations of fish in deep pools may facilitate contagion (Johnsen and Jensen, 1994).

There is probably no one way in which *A. salmonicida* enters a susceptible host, but the bacterium is transferred readily by the horizontal contact among infected fish that are cohabited with naive individuals (Nordmo *et al.*, 1998a). Although some workers have failed to transmit the disease via a gastrointestinal route (Krantz *et al.*, 1964; McCarthy, 1977b), Klontz and Wood (1973) observed clinical furunculosis in sablefish (*Anaplopoma fimbria*) that resulted from ingestion of infected coho salmon (*O. kisutch*). Translocation across the foregut due to the detachment of enterocytes caused by exposure to this pathogen is a probable route of bacterial infection (Ringø *et al.*, 2004; Jutfelt *et al.*, 2006). Even if the bacterium enters the peritoneum, its survival may not be assured. Garduno *et al.* (1993a) found that *A. salmonicida* might be lysed by a soluble component in peritoneal fluid. In addition, Miyazaki and Kubota (1975a,b) also provided histopathological evidence that supported both perbranchial and percutaneous routes of infection.

Present information suggests that the source and reservoir of infection and transmission of atypical *A. salmonicida* are probably similar to those for typical strains (Wiklund and Dalsgaard, 1998). Tuffery and Dehand (1979) were not able to isolate bacteria from tissues of live or dead carp that had been injected with a carp erythrodermatitis variant of *A. salmonicida*. Elliott and Shotts (1980b) also found that goldfish injected with *A. salmonicida* isolated from clinical cases of Goldfish Ulcer Disease died without developing ulcers. However, ulcers were produced if the goldfish were exposed to the pathogen either in the water or after scarification in which a sample of scales was removed and the exposed dermis was swabbed with the inoculated bacteria. In studies conducted at the National Fish Health Research Laboratory, one of us (RC) also found that rainbow trout, brook trout (*Salvelinus fontinalis*) and Atlantic salmon died after injection with atypical variants of *A. salmonicida* isolated from goldfish, without the development of dermal ulcerations. Removal of mucus, followed by skin inoculation, produced ulcers in brook trout and Atlantic salmon, but not in rainbow trout. Atypical isolates from ulcerated flounders (*Platichthys flesus*) could reproduce dermal ulcerations only if the bacterium was injected subdermally or if the skin of fish had been scarified prior to waterborne challenge (Wiklund, 1995a). The importance of dermal scarification is further emphasized by Takahashi *et al.* (1975), who concluded that the natural transmission of Goldfish Ulcer Disease was most severe among fish whose dermis had been eroded severely either by ectoparasites or by handling.

A. salmonicida has been isolated from the sea louse, *Lepeophtheirius salmonis* (Nese and Enger, 1993) in the marine environment and from *Argulus corregoni* (Shimura *et al.*, 1983) and *Tetrahymena pyriformis* (King and Shotts, 1988) in the freshwater environment. Because each of these parasites is capable of inducing severe branchial or dermal damage, it has been theorized, but not substantiated, that infected ectoparasites may act as vectors in the spread of furunculosis. Infestations produced by the digenetic trematode, *Diplostomum spathaceum*, have also been shown to increase the prevalence of atypical *A. salmonicida* infections in grayling (*Thymallus thymallus*) hearts (Pylkko *et al.*, 2006).

Horizontal transmission of *A. salmonicida* has also been demonstrated where

molluscs have been co-cultured with susceptible hosts (Bjoershol *et al.*, 1999; Starliper, 2001). Ebony shell mussels (*Fusconaia ebena*) can amass significant levels of *A. salmonicida* when cohabited with infected brook trout (Starliper, 2005). Concentrations of *A. salmonicida* were as high as 1.8×10^5 cfu/g in soft tissue homogenates and 2.8×10^5 cfu/ml in body fluids. Under these concentrations, it took approximately 15 days until the pathogen was depurated to the extent that the mussels no longer served as reservoirs of infection. Another novel application of husbandry techniques that may effect the transmission of infection involves the use of wrasse as cleaner fish to clear sea lice from Atlantic salmon in net-pen culture. The wrasse may themselves become infected with atypical variants either by ingesting infected sea lice or through horizontal exposures with other infected fish. Infected wrasse may, therefore, act as a further reservoir of contagion for salmon with which they are co-cultured (Collins *et al.*, 1991; Laidler *et al.*, 1999). As a consequence of such infection, it has been suggested that wrasse should not be released into the environment or transferred between facilities at the end of specific production cycles (Treasurer and Laidler, 1994).

In addition to horizontal transmission, Wooster and Bowser (1996) found that viable *A. salmonicida* could be dispersed in aerosol droplets at least 104.1 cm in the air. Viable *A. salmonicida* was also recovered from the water of tanks that were exposed to an aerosol spray downwind from the contaminant source. This study indicates that aerosol transmission of *A. salmonicida* may be particularly problematic where culture systems use spray bars within raceways, and especially in experimental aquaria arranged in close proximity. Aerosol transmission may also be enhanced by the fact that concentrations of *A. salmonicida* are enriched in the lipid-rich surface microlayer at the air–water interface (Enger *et al.*, 1992).

Mackie *et al.* (1930) noted the presence of *A. salmonicida* within the ovaries and testes of infected fish, but failed to induce intraovum infections experimentally and transmit furunculosis vertically from parent to offspring. Consequently, they concluded that *A. salmonicida* contaminated the surface of the eggs and recommended that such eggs should be disinfected to minimize or prevent further contagion (Mackie *et al.*, 1933). McCarthy (1977b) indicated that vertical transmission was not a significant route of infection because *A. salmonicida* from infected parents were unlikely to survive in the eyed-egg stage. Similarly, Bullock and Stuckey (1987) were unable to document vertical transmission of furunculosis among the progeny of parental stocks that either had survived furunculosis epizootics or had been injected experimentally with *A. salmonicida* prior to spawn.

McCarthy (1977b) determined that the bacterium survived 24 days in natural brackish water, 17 days in non-sterile fresh water and 8 days in seawater. He also found that *A. salmonicida* survived on wet or dry nets for 6 days and for 32 days in the tissues or carcasses of fish that had died from furunculosis. According to Rose *et al.* (1990a), *A. salmonicida* remains viable for generally less than 10 days, which is consistent with the obligate nature of this pathogen. Deere *et al.* (1996a) found that culturable cells could not be detected after 10 days in distilled water, but persisted for 33 days in lake water. On fish husbandry equipment, the bacterium may adhere more preferentially to plastics than it does to stainless steel (Carballo *et al.*, 2000). Sakai (1986) had also shown that virulent strains survived for more than 15 weeks in humic acid, tryptone and cleaned river sand, but were not viable within 5 weeks in the absence of sand.

Although viable counts of *A. salmonicida* could not be detected after 4 weeks in loamy sediment and after 7 weeks in sandy sediment, naked DNA and DNA from released cells were still present after 13 weeks (Deere *et al.*, 1996b). Intact cells of *A. salmonicida* were observed microscopically even when the bacterium could not be cultured on agar plates (Morgan *et al.*, 1993; Effendi and Austin, 1994). These NCBV cells sustained morphological changes that resulted in smaller size and loss of

pathogenicity for Atlantic salmon (Effendi and Austin, 1995). Such cells were considered to be dormant (Ferguson *et al.*, 1995).

Rose *et al.* (1990b) argued against dormancy and suggested that a small number of cells (below detection by culture) remained viable and could be resuscitated by the addition of nutrient broth. Pérez *et al.* (1995) also supported the argument against dormancy, indicating that some *A. salmonicida* remained viable in filtered seawater for 23–90 days at 10°C and for 12–69 days at 20°C. After the addition of nutrients, Husevåg (1995) showed that a small percentage of *A. salmonicida* cells, previously starved for 6 months in a nutrient-free medium at 15°C, could be revived in liquid but not on agar media. Pickup *et al.* (1996) found that the addition of arginine and methionine into microcosms seeded with *A. salmonicida* postponed onset of a non-culturable state, but the cells still showed morphological changes (e.g. reduced size). Whether or not *A. salmonicida* enters a true dormancy, it is evident that nutrient flow within the environment can rejuvenate the growth and replication of this bacterium so that even a small number of non-culturable cells may become a potential reservoir of infection for naive fish. Wiklund (1995b) found that cytochrome oxidase-negative isolates from flounders in the Baltic Sea survived for short periods in microcosms containing sterile brackish water, but persisted for as long as 63 days in microcosms containing sterile brackish water and sediment. Hiney *et al.* (2002) recorded that *A. salmonicida* was not cultured within 0.3 days from acidic microcosms containing sediments rich in humic acids, whereas the bacterium was cultured readily for up to 40 days from neutral microcosms containing low levels of humic acids. Although Stanley *et al.* (2002) found that they could detect *A. salmonicida* using ELISA and PCR assays long after they could be isolated from water–sediment microcosms, these bacteria did not induce disease in test fish. Hence, induction of furunculosis was observed only when the bacterium was cultured.

Diagnostic Methods

Presumptive diagnosis of motile aeromonad septicaemias may be based on the species of fish affected, the past disease status of those fish and the presence of clinical signs of the disease. However, bacteria must be isolated and identified biochemically to provide a definitive diagnosis. Either tryptic soy agar (TSA) or brain–heart infusion agar (BHIA) is a suitable medium for the primary isolation of motile aeromonads from diseased fish. Because mixed bacterial infections are common in fish affected with haemorrhagic septicaemias, it is often difficult to isolate pure cultures of a single species of motile aeromonads from clinically diseased tissues. Isolates should be Gram-negative, motile rods that are cytochrome oxidase positive, ferment glucose and are insensitive to the vibriostatic agent 0/129 (2,4-diamino, 6,7-di-isopropyl pteridine). In addition, the bacteria produce 2,3-butanediol and reduce nitrate to nitrite. Glucose fermentation is a critical reaction that differentiates the motile aeromonads from glucose-inert or oxidative *Pseudomonas* spp. (Bullock, 1961; Walters and Plumb, 1978). Although most strains of *A. hydrophila* produce gas during the fermentation of glucose, some motile aeromonads isolated from diseased fish are anaerogenic (Ross, 1962).

In order to facilitate the recovery of motile aeromonads from fish tissues upon primary isolation, Shotts and Rimler (1973) designed the differential medium, RS agar. The medium is prepared with the following ingredients (g) dissolved in distilled water to a volume of 1 l l-lysine hydrochloride (5.0), l-ornithine hydrochloride (6.5), l-cystine hydrochloride (0.3), maltose (3.5), sodium thiosulfate (6.8), bromothymol blue (0.03), ferric ammonium citrate (0.8), sodium deoxycholate (1.0), novobiocin (0.005), yeast extract (3.0), sodium chloride (5.0) and agar (13.5). The mixture is boiled for 1 min and brought to pH 7.0. The agar should be incubated at 37°C for 48 h to ensure optimal differentiation of bacteria. Colonies of motile aeromonads are yellow; those of *Pseudomonas*, *Escherichia* and

Enterobacter are green; and those of *Edwardsiella* are green with black centres. Although *Proteus vulgaris* and some *Citrobacter* are also yellow on RS agar, these colonies have black centres, indicative of their ability to produce hydrogen sulfide. The presence of yellow colonies should not be accepted as a definitive diagnosis. *A. salmonicida* will also produce yellow colonies on RS but, unlike the motile aeromonads, growth of this bacterium is inhibited at 37°C. Davis and Sizemore (1981) also indicated that RS supported growth of a limited number of yellow colonies whose DNA homology ratios were inconsistent for the genus *Aeromonas*. Many of these isolates, especially those obtained from low-salinity sites, were also identified by the API 20E system as *A. hydrophila*. Therefore, additional biochemical tests should be performed on isolated colonies that have been cloned from purified colonies on either TSA or BHIA. It is important to note that reactions inconsistent for the species can occur if one uses colonies picked directly from RS agar. For example, Overman *et al.* (1979) found that 8% of the isolates they examined were cytochrome oxidase negative when assayed directly from this medium.

A more selective starch–glutamate–ampicillin–penicillin-based medium (SGAP-10C) has been developed for isolation of motile aeromonads from water samples (Huguet and Ribas, 1991). To improve selectivity, ampicillin (20 mg/l) is added to reduce pseudomonads together with 10 μg/l of C (25 μg) from glucose, which allows restoration of 'stressed' aeromonads. The absence of NaCl also inhibits growth of halophilic vibrios. Growth of motile aeromonads was optimal on SGAP-10 when the medium was incubated at 24°C for 48 h. Colonies were approximately 3 mm in diameter. They were rounded, smooth and slightly raised with entire margins, and were characteristically pink, while the medium within their immediate area appeared red. In clinical situations, Jenkins and Taylor (1995) greatly preferred the enhanced selectivity of SGAP-10 to that of RS.

Because motile aeromonads are ubiquitous and have considerable antigenic diversity, serodiagnostics have limited value. Agglutination procedures (Shin *et al.*, 2000), fluorescent antibody tests (Eurell *et al.*, 1978; Soltani and Rabani Khorasgani, 1999) and immunoenzyme assays (Lewis, 1981; Sendra *et al.*, 1997; Mishra, 1998; Shome *et al.*, 2005) have been adapted for use and are most effective within homologous antigen/antibody systems. These assays may detect accurately strains of motile aeromonads against which homologous detection antibodies have been developed, but detection of heterologous strains makes serodiagnostic identification impractical. Fliermans and Hazen (1980) found that a total of three different antisera to *A. hydrophila* yielded positive fluorescent antibody reactions with only 27.5% of the *A. hydrophila* isolates that they examined. Therefore, the lack of an effective polyvalent antiserum specific for *A. hydrophila* limits reliable serodiagnostic detection of this pathogen. Calabrez *et al.* (1993) developed a polymerase chain reaction (PCR) technique that was used to detect strains of *A. sobria* in water, sediments and fish. Genetic analysis suggests that there are presently 14 genospecies recognized within the aeromonads (Joseph and Carnahan, 1994) and, consequently, a number of species-specific PCR tests would need to be developed and run on individual clinical samples in order to achieve accurate diagnosis. Rahman *et al.* (2005) developed primers, designated Aerom-Fa and Aerom-Ra, which yielded a 1206 bp product from the DNA of all of the recognized *Aeromonas* genospecies. DeteNam and Joh (2007) have developed a hexaplex-PCR assay that detects six aeromonad virulence factors: aerolysin, GCAT, serine protease, nuclease, lipase and lateral flagella. Using this assay, the investigators examined culture water and diseased fish on a seasonal basis. They found that *A. sobria* was the dominant aeromonad and that the dominant virulence factors produced during all seasons were aerolysin and nuclease.

Presumptive diagnoses of typical and atypical variants of *A. salmonicida* from clinically diseased or moribund fish are

based on clinical indications of disease, the type and number of species infected and the clinical history of disease within the affected population or facility. Presumptive diagnosis of atypical *A. salmonicida* infections in salmonids or non-salmonids is more difficult than the diagnosis of typical furunculosis, because clinical signs vary and dermal lesions are often contaminated with opportunistic fungi and bacteria. Definitive diagnosis of all *A. salmonicida* infections requires isolation and identification of the causative bacterium. Pigmented typical *A. salmonicida* strains can be isolated readily from kidney tissues of dead or moribund fish using commercial media such as TSA or BHIA. Differentiation of colonial types that grow on primary isolation can be facilitated by the simple addition of 0.1% (weight: volume) Coomassie Brilliant Blue (CBB) R-250 into either of the aforementioned media. When cultured on CBB agar, the A-layer protein that is present in virulent strains of *A. salmonicida* will absorb the CBB, a protein-specific dye. Consequently, virulent *A. salmonicida* develop dark blue to deep violet friable colonies on CBB agar (Cipriano and Bertolini, 1988; Cipriano and Blanch, 1989). Although CBB agar facilitates presumptive identification, it is not a selective medium and a definitive diagnosis should be confirmed via additional biochemical tests (Teska and Cipriano, 1993). Isolates of *A. salmonicida* subsp. *salmonicida* from salmon in Yamagata Prefecture (Japan) did not autoagglutinate in broth or produce A-protein and appeared as greyish to white colonies on CBB agar (Nomura *et al.*, 1994). The authors concluded that these aberrant characteristics might have been induced by elevated incubation temperatures. Moki *et al.* (1995) found that *A. salmonicida* isolates cultured at 15 and 20°C autoagglutinated, but 40% of colonies incubated at 25°C did not. These observations suggest that *A. salmonicida* be isolated and cultured at temperatures that do not exceed 20°C in order to retain their ability to autoagglutinate, retain A-protein and produce blue colonies on CBB agar.

Atypical pigmented or non-pigmented strains of *A. salmonicida* are fastidious on initial isolation from lesions and the presence of biotic contaminants may interfere with an accurate diagnosis. McCarthy (1977a) suggested, therefore, that isolation should be attempted from early and advanced lesions using a minimum of six fish from each epizootic. Elliott and Shotts (1980a) found that primary isolation of atypical isolates causing Goldfish Ulcer Disease was facilitated on blood agar. Additionally, Bootsma *et al.* (1977) suggested that diagnosis of Carp Erythrodermatitis should be enacted, first, by infecting healthy common carp experimentally by contact with diseased carp and, second, by inoculation of lesion material from experimentally infected carp on to a medium consisting of tryptose blood agar base (Difco), 10% blood serum and filter decontaminated ampicillin and polymyxin B sulfate to final concentrations of 50μg/ml and 50IU/ml, respectively. These fastidious atypical isolates have a specific requirement for haem that is essential for the initiation of growth, especially when the bacteria are in low concentrations in the initial inoculum. Incorporation of haematoporphyrin and haemoglobin into the medium may, therefore, provide a suitable source of haem and increase plating efficiency (Ishiguro *et al.*, 1986).

Following primary isolation, purified clones of typical and atypical strains of *A. salmonicida* are confirmed readily if resultant bacteria are Gram-negative, non-motile rods that are cytochrome oxidase positive, ferment glucose and differentiate according to the biochemical criteria listed in Tables 12.1 and 12.2. The production of a brown water-soluble pigment remains a principal phenotypic characteristic in the presumptive identification of most *A. salmonicida* subsp. *salmonicida*. Griffin *et al.* (1953) noted that the incorporation of L-phenylalanine and L-tyrosine in media (pH=6.5–6.8) enhanced pigmentation. However, pigmentation was delayed in the presence of fermentable sugars (e.g. maltose), by addition of 10% carbon dioxide, by simply tightening the caps of test tubes, or by incubation of cultures above 22°C (Griffin, 1953). The incorporation of 0.1% glucose in bacteriological media may not only inhibit pigmentation but also cause an overall reduction in the size of the

bacterial colonies (Altman *et al.*, 1992). Although the presence of brown pigment may facilitate diagnosis, similar pigments are also produced by other aquatic aeromonads (Ross, 1962) and pseudomonads (Hamilton-Miller, 1975; Altman *et al.*, 1992).

Dalsgaard *et al.* (1998) noted that the identification of atypical isolates was particularly troublesome unless biochemical tests were interpreted within a standardized timeframe. It is also important to note that some strains of *A. salmonicida* may be cytochrome oxidase negative; a result that is inconsistent for this species (Chapman *et al.*, 1991; Pedersen *et al.*, 1994; Wiklund *et al.*, 1994).

A number of different serological techniques may be used for identification (Rabb *et al.*, 1964; McCarthy and Rawle, 1975; McCarthy and Whitehead, 1977; Eurell *et al.*, 1979). Sakai *et al.* (1986) noted that enzyme immunoassays (ELISA) amplified with secondary and tertiary antibodies might detect as little as single-digit colony-forming units (CFUs) of *A. salmonicida* per ml. They also found that normal ELISA and immunofluorescence assays detected a minimum of 10^2 and 10^3 cfu of the pathogen, respectively; but it required as many as 10^7 cfu for detection using either latex agglutination or coagglutination procedures. Adams and Thompson (1990) also demonstrated that the threshold limit for detection of *A. salmonicida* using a normal ELISA test was approximately 10^3 cfu/ml. ELISA has also been used as a confirmatory diagnosis for bacteria cultured from clinical specimens, as long as the cultured bacterium has a colonial morphology that is consistent for *A. salmonicida* and produces both brown pigment and cytochrome oxidase (Bernoth, 1990). Gilroy and Smith (2003) also found it possible to detect as little as $1–4\times10^3$ cfu/ml of *A. salmonicida* in spiked environmental matrices, such as water and sediments.

Fish may be covert carriers of *A. salmonicida* even when culture or other methods of diagnosis are negative (Hiney *et al.*, 1997). In such cases, the stress-induced furunculosis test as described by Bullock and Stuckey (1975) and modified by McCarthy (1977b) has proven to be the most reliable procedure for detection of *A. salmonicida* in carrier fish (Hiney *et al.*, 1994; Cipriano *et al.*, 1997). Briefly, fish are injected with 20 mg of prednisolone acetate/kg of fish and maintained for 14 days in water at 18°C. All mortality is cultured to confirm the presence or absence of *A. salmonicida*. If no fish die, the observation period may be extended or all fish may be sacrificed and cultures prepared from their kidneys. Stress-inducible furunculosis may be seasonal or transient in nature and can be enhanced by certain physiological (Scallan and Smith, 1993) and environmental stressors (Scallan *et al.*, 1994). Filtration (Maheshkumar *et al.*, 1990; Ford, 1994) or PCR (O'Brien *et al.*, 1994) analyses may also be adapted to detect and monitor concentrations of *A. salmonicida* in culture water or facility effluents.

Molecular-based techniques offer the possibility of progressing from culture-dependent to culture-independent approaches without the need to kill animals. A number of PCR assays have been developed that can be used either to achieve diagnosis of furunculosis directly from clinically infected samples (Gustafson *et al.*, 1992; Calabrez *et al.*, 1993; Mooney *et al.*, 1995; Høie *et al.*, 1997) or as a molecular tool to supplant biochemical confirmation following primary isolation (Hiney *et al.*, 1992; Miyata *et al.*, 1996; Oakey *et al.*, 1998, Byers *et al.*, 2002a,b). Hiney *et al.* (1992) designed PCR primers (PAAS) that amplified a 423 bp DNA fragment specific to *A. salmonicida*. In the resultant PCR assay, 10 fg of total DNA produced a positive signal after 4 h of autoradiography; a result that was equivalent to the DNA content of 2.4 *A. salmonicida* cells. By modifying the assay to remove PCR-humic inhibitors, often associated with environmental samples, the assay yielded positive detection of the pathogen in hatchery effluents and faeces from clinically diseased or covertly infected fish (O'Brien *et al.*, 1994). It also detected low numbers of non-culturable but morphologically intact cells of *A. salmonicida* in lake water (Morgan *et al.*, 1991). Gustafson *et al.* (1992) also amplified a 421 bp (PA) sequence from the 3′ region of the A-protein gene (*vapA*), which provided specific and sensitive

detection at less than 10 cfu/mg of tissue. Miyata *et al.* (1996) also designed an MIY PCR that amplified a 512 bp product specific for *A. salmonicida* subsp. *salmonicida*, but negative for *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida* or other atypical *A. salmonicida* isolates. Oakey *et al.* (1998) used RAPD-PCR to identify a single RAPD-PCR fragment (designated 15e) that was common to *A. salmonicida*, but comprised five DNA fragments of similar size that differed in nucleotide sequences. DNA probes were developed from these fragments. Two probes provided specific detection of the four current subspecies of *A. salmonicida*, as well as other atypical isolates. Another probe hybridized with the subspecies *salmonicida*, *achromogenes* and *masoucida*. A fourth probe hybridized with subspecies *salmonicida* and *achromogenes*, and a fifth probe hybridized with subspecies *salmonicida*, *achromogenes* and *smithia*.

Byers *et al.* (2002a) found that using both the PAAS and PA assays provided the most complete identification of *A. salmonicida*. The AP and PAAS assays detected *A. salmonicida* easily from gills, mucus and kidneys of overtly diseased fish, but was less reliable than culture when used among covertly infected, asymptomatic salmonids (Byers *et al.*, 2002b). Beaz-Hidalgo *et al.* (2008) recently developed primers (Fer3, Fer4) from a fragment of the *fstA* gene which encodes for the ferric-siderophore receptor of *A. salmonicida* and found that their PCR was superior to culture in detection of *A. salmonicida* from asymptomatic fish. Real-time PCR using self-quenched primers labelled with a single fluorophore was also produced by Balcazar *et al.* (2007a); it provides sensitive species-specific detection and quantifies *A. salmonicida* in fish tissues.

Because PCR assays may often detect concentrations of bacteria below those obtained by culture, predictive validation and interpretation of results in the absence of definitive culture-positive results are often problematic (Hiney and Smith, 1998). In an attempt to validate the detection results obtained using ELISA and PCR, Smith *et al.* (2003) examined feral salmonids and the sediments in their habitats. None of the 223 kidney samples were positive by culture, whereas 23.3% were positive using ELISA and 20.6% were positive using PCR. Unfortunately, there was only 27% agreement in the results obtained using both proxy methods. Among 35 sediment samples, 53% were positive using ELISA and 33% using PCR, with only 24% concordance. These results demonstrate the difficulties inherent in validating the results produced using proxy assays (e.g. ELISA and PCR) among natural samples, when culture is negative.

Genome Structure and Transcription

The definitive genome structure of *A. salmonicida* or any of the fish pathogenic motile aeromonads is as yet not available. A physical map of the circular 4658±30 kb chromosome of *A. salmonicida* A449 was constructed using pulsed field gel electrophoresis (PFGE) and served to give the approximate location of some virulence genes, which were not clustered together (Umelo and Trust, 1998). By comparing the I-Ceul genomic digest fingerprint of typical and atypical isolates, the homogeneity of the former but diversity of the latter was demonstrated. Other studies have focused on specific genes, particularly those concerned with virulence, expressed by the pathogen, whereas other investigators have emphasized the host response. The species-specific structural gene for the monomeric form of the A-layer protein of an atypical strain of *A. salmonicida* was cloned into a pET-3d plasmid to express a recombinant protein in *E. coli* BL21(DE3). The induced monomeric protein was purified using ion exchange chromatography on Q-Sepharose. The recombinant protein was undistinguishable from the wild-type protein when examined by SDS-PAGE and gel filtration chromatography. The A-protein activated the secretion of tumour necrosis factor cu from murine macrophages (Maurice *et al.*, 1999).

The *vapA* gene of *A. salmonicida*, which encodes the subunit of the A-layer, has been cloned and sequenced (Trust

et al., 1996). Nucleotide sequence analysis of the 374 bp of DNA immediately upstream of *vapA* revealed two possible promoter sequences and other regulatory sequences (Chu *et al.*, 1993). Sequencing and PCR showed that the region was conserved in wild-type *A. salmonicida* (Chu *et al.*, 1993). The LPS *O*-polysaccharide side chains of *A. salmonicida* are homogeneous in length and their synthesis and secretion involves the product of the *abcA* gene (Trust *et al.*, 1996). Mutagenesis of the *asoA* gene results in the disruption of the outer membrane and A-layer, which results in an increase in systemic virulence following intraperitoneal application of the bacteria (Trust *et al.*, 1996). Conversely, mutagenesis of the *asoB* gene resulted in effects on translocation of the A-protein across the cytoplasmic membrane. Trust *et al.* (1996) reported that a highly conserved porin from *A. salmonicida* elicited protective immunity when administered intraperitoneally to rainbow trout.

The type III secretion system (TTSS) of *A. salmonicida* subsp. *salmonicida* has been associated with virulence. Dacanay *et al.* (2006) deleted three TTSS effector genes, i.e. *aexT*, *aopH* and *aopO*, and *ascC*, which encoded the outer membrane pore of the secretion apparatus. An *ascC* mutant was avirulent by intraperitoneal injection and immersion in Atlantic salmon, but did not confer protection from subsequent challenge with the parental virulent culture. H-1 NMR spectroscopy of plasma showed significant differences in the metabolite profiles between the animals exposed to the parental culture or the *ascC* mutant. The conclusion was that whereas TTSS was necessary for virulence, removal of individual effectors exerted negligible effect on virulence but did have significance on colonization of Atlantic salmon (Dacanay *et al.*, 2006).

The *exe* gene cluster, which is a member of the pul-related operon family required for signal sequence-dependent secretion of proteins, was cloned from *A. salmonicida* in the broad host range cosmid, pLAFR3. Twelve genes, i.e. *exec–exeN*, were identified using partial or complete nucleotide sequence analyses. On alignment of *A. salmonicida* and *A. hydrophila exe* sequences, a 73 bp so called 'silent' deletion was identified near the end of the *A. salmonicida exeF* gene. It is noteworthy that a gene-encoding prepilin peptidase, i.e. the PulO homologue, is in this region. Evidently, the *exeN* gene is the last gene of this operon, and it is followed by an ORF encoding a putative transcription regulator (Karlyshev and MacIntyre, 1995). Tsoi *et al.* (2003) used a commercially available human cDNA microarray to study the expression of genes in the livers of Atlantic salmon which had been infected with *A. salmonicida*. cDNA probes were also prepared from the total RNA from livers of infected and uninfected Atlantic salmon by reverse transcription and hybridized to human GF311 microarrays. Of 4131 genes included on the microarray, 241 spots gave signals using labelled RNA from the liver of uninfected fish. Four of these spots gave a greater than twofold increase in infected Atlantic salmon. These upregulated genes were ADP/ATP translocase, sodium/potassium ATPase, acyloxyacyl hydrolase and platelet-derived growth factor (PDGF-A). A search of the BlastN database revealed an AAT2 homologue from Atlantic salmon, and a reverse transcriptase-PCR (RT-PCR) using primers based on this sequence confirmed upregulation, i.e. ~ 1.8-fold during the early stage of infection (Tsoi *et al.*, 2003). Customized Atlantic salmon cDNA microarrays comprising >4000 amplicons were used to investigate the response of the head kidney, liver and spleen to infection by *A. salmonicida* (Ewart *et al.*, 2005). RT-PCR was used to confirm the expression of 12 genes encoding components of innate immunity, with the infection process and with no known homologue, which were identified by the microarrays (Ewart *et al.*, 2005).

Virulence Factors

The ability of a pathogen to locate, attach to and subsequently infect a susceptible host is a primary step in the development of disease. Motile aeromonads that have been cultured from lesions display a greater chemotactic response to skin mucus than

isolates that are obtained as free-living organisms from pond water (Hazen *et al*., 1982). Ascencio *et al*. (1998) showed that *A. hydrophila*, *A. caviae* and *A. sobria* adhered to animal cell lines that had mucous receptors, and that the proportion of *A. hydrophila* which bound mucins was greater than the proportions of *A. caviae* and *A. sobria*. Trust *et al*. (1980b) also indicated that *A. hydrophila* had adhesive agglutination characteristics which facilitated attachment to eucaryotic cells. Motile aeromonads produced fimbriae (pili) that facilitated adhesion, but these structures were common to cells regardless of strain virulence (delCorral *et al*., 1990). Lee *et al*. (1997) actually found two groups of adhesins in *A. hydrophila*. The major group consisted of high molecular weight proteins, and the largest component was a 43-kDa outer membrane porin. A minor group of adhesins was also identified that consisted of low molecular weight proteins, but these were less effective in mediating bacterial adhesion and invasion into carp epithelial cells. Fang *et al*. (2004) sequenced the gene of the 43-kDa major adhesin (designated as AHA1) from *A. hydrophila* that had an open reading frame encoding a polypeptide of 373 amino acids with a 20 amino acid putative signal peptide. Western blot analysis showed that this adhesin was conserved among various strains and an efficacious immunogen. When *A. hydrophila* was grown under conditions that supported capsular formation, those cells adhered slightly better to fish cell lines than others that were not capsulated (Merino *et al*., 1997). These capsulated forms also invaded fish cell lines more readily than those that were not capsulated.

In addition to adhesins, Dooley and Trust (1988) characterized a tetragonal surface 52-kDa protein array among virulent isolates of *A. hydrophila*. This S-layer was also reported by Ford and Thune (1991) among motile aeromonads that were isolated from clinically diseased catfish. The existence of such layers generally increases cellular hydrophobicity, which enhances bacterial resistance to complement-mediated lysis and phagocytosis by leucocytes. Gado (1998) noted that virulent aeromonad isolates from tilapia shared a common resistance to the killing effect of tilapia serum, but Sakata and Shimojo (1991) found that serum resistance was not always consistent with the presence of the S-layer. Leung *et al*. (1995) also found that virulent *A. hydrophila* strains from diseased fish in South-east Asia were resistant to the bactericidal activity of normal serum, but other factors such as the lack of autoagglutination in 0.2% acriflavine, instability after boiling, production of an S-layer, proteases and haemolysins did not necessarily correlate with virulence. Byers *et al*. (1986) have also shown that *A. hydrophila* can produce siderophores that confer resistance against the capacity for serum transferrin to inhibit bacterial growth.

When injected intraperitoneally into carp and goldfish, *A. hydrophila* starved for 24 h in either FW2 (0.60% NaCl, 0.5% KCl, 0.1% $CaCl_2$ and 0.2% $MgCl_2$ in distilled water) or 0.85% NaCl were more virulent than those cultured in nutrient broth (Rahman *et al*., 1997). Starvation of *A. hydrophila* causes shifts in outer membrane and S-layer protein patterns that seemingly impart enhanced resistance to phagocytosis by macrophages (Rahman *et al*., 1998). Starved bacteria adhered to the skin of Crucian carp (*C. cuvieri*) in significantly greater numbers than cells cultured in nutrient broth (Rahman and Kawai, 1999), and these cells were also more resistant to bactericidal activities resident within dermal mucus and serum.

Many studies have attempted to delineate other virulence mechanisms of motile aeromonads further, which is complicated by the diversity of extracellular proteases produced by these organisms (Nieto and Ellis, 1991). Kou (1973) found that many virulent, avirulent and attenuated aeromonads possessed haemorrhagic factors and lethal toxins whose activities were greater in virulent bacteria versus avirulent or attenuated counterparts. Olivier *et al*. (1981) indicated that both *A. hydrophila* and *A. sobria* produced enterotoxins, dermonecrotic factors and haemolysins. Although both species produced haemolysis on blood agar plates at 30°C, only *A. hydrophila* did so at 10°C. Because these researchers were

working with salmonid fish, they suggested that the haemolysis of erythrocytes by *A. hydrophila* at temperatures comparable to those of the water in which fish lived may at least have partially accounted for the difference in virulence between *A. hydrophila* and *A. sobria*. The combination of strongly pronounced haemolytic and proteolytic activities was evident in 90% of highly pathogenic *A. hydrophila* and 87.5% of *A. sobria* isolates obtained from carp (Rogulska *et al.*, 1994). Gonzalez-Rodriguez *et al.* (2004) found that *A. hydrophila* produced haemolysin in salmon extracts at 28°C but not at 4°C, whereas proteolytic activity was only expressed at 28°C. By contrast, *A. veronii* biovar *sobria* produced both haemolysin and proteolytic activity at 28°C in salmon extracts. Esteve *et al.* (1995) determined that most *A. hydrophila* and *A. jandaei* isolates were highly pathogenic for eels and produced elastases and haemolysins. Mateos *et al.* (1993) found that the production of haemolysins, caseinases and elastases decreased sharply when environmental strains of *A. hydrophila* were cultured at 37°C, but cytotoxin production was only slightly less at 37°C than it was at 28°C. For the most part, Uddin *et al.* (1997) found that optimal temperatures for protease production (27.6+/−4.9°C) were lower than those that favoured optimal growth (34.5+/−1.0°C).

Enterotoxins, haemolysins, proteases, haemagglutinins and endotoxins produced by this complex of bacterial organisms have been the subject of much research (Cahill, 1990). However, the composite nature of virulence observed both inter- and intraspecifically among the motile aeromonads may be best defined by looking at synergistic relationships between virulence factors. For example, Rigney *et al.* (1978) found that individual injections of either endotoxin or haemolysin alone did not produce clinical pathology in frogs. When both endotoxin and haemolysin were injected together, frogs exhibited clinical signs of red leg disease. Thune *et al.* (1982a) also found that channel catfish were tolerant to injections of endotoxin at concentrations ≥400 μg endotoxin in 7.2 g fish, but extracellular cell-free extracts produced an LD_{50} value of 15.7 μg protein within 48 h. Thune *et al.* (1982b) later showed that this extract was proteolytic, but not haemolytic. Chabot and Thune (1991) then characterized three proteases produced by *A. hydrophila*: a heat-labile serine protease (optimum pH=7.5), a heat-stable metalloprotease (optimum pH=8.0) and a moderately heat-stable metalloprotease (optimum pH 7–11). Esteve and Birkbeck (2004) noted that haemolysins were produced during logarithmic growth and decreased quickly thereafter. By contrast, proteases prevailed in supernatants of stationary cultures, at which time a 68-kDa serine protease was four times greater than that of the metalloprotease. Rodriguez *et al.* (1992) purified a metalloprotease, a serine protease and a haemolysin from culture supernatants of *A. hydrophila*. Each was lethal for rainbow trout. The metalloprotease had a molecular weight of 38 kDa, was stable at 56°C for 10 min, was not cytotoxic but produced an LC_{50} value of 150 ng/g of trout. The serine protease had a molecular weight of 22 kDa, was stable at 56°C for 10 min, possessed cytotoxic activity and had an LC_{50} value of 150 ng/g of trout. The haemolysin was alpha-haemolytic, had a molecular weight of 68 kDa and produced an LC_{50} value of 2 μg/g of trout. The haemolysin was stable at 56°C for 20 min and at 60°C for 10 min. Because the haemolysin possessed esterase activity on beta-naphthyl acetate, Rodriguez *et al.* (1992) suggested that the compound might actually be different from either alpha- or beta-haemolysin. Cascón *et al.* (2000) cloned an *A. hydrophila* gene (*ahyB*) that encoded elastolytic activity. They found that AhyB was synthesized as a pre-protein that was further processed into protease and a C-terminal pro-peptide. The protease hydrolysed casein and elastin and displayed high similarity to metalloproteases.

Allan and Stevenson (1981) observed that aeration increased growth rates, cell yield and the amount of proteolytic activity in culture supernatants. Proteolytic activity decreased, however, when cultures were incubated at 37°C. Furthermore, extracts from a protease-deficient mutant were more

toxic to fish than similar extracts from isogenic wild strains. Because the extract from the protease deficient mutant had a marked increase in haemolytic activity, these workers concluded that haemolysin, not protease, was the principal virulence factor of *A. hydrophila*. The results of other studies, however, might caution against such conclusions. Rogulska *et al.* (1994) found that haemolytic activity was high in 93% of very pathogenic strains of *A. hydrophila* and the activity was also high in 87.5% of pathogenic *A. sobria*; however, haemolytic activity alone was not a decisive factor because even some (15%) of the avirulent *A. hydrophila* isolates were strongly haemolytic. The *A. hydrophila* β-haemolysin gene (*AHTPS30HEM*) has been cloned and shows little homology with those from *A. sobria*, *A. trota* and *A. salmonicida* (Xia *et al.*, 2004).

The haemolytic toxin, aerolysin, produced in culture supernatants of *A. hydrophila* (Bernheimer and Avigad, 1974) is a 51.5-kDa protein that is a composite of two components that have pIs of 5.39 and 5.46 by isoelectric focusing (Buckley *et al.*, 1981). Enterotoxic activity expressed by a cytotoxic enterotoxin was detected in a culture filtrate of *Aeromonas* sp. after its cytotoxic activity was neutralized by monoclonal antibodies against homologous aerolysin (Chopra *et al.*, 1992). *Aeromonas* spp. secrete an aerolysin precursor into the culture medium, where it is activated by proteolytic removal of a C-terminal fragment. Conversion to active aerolysin is effected by treating the precursor protein with either trypsin or extracellular protease produced by *Aeromonas* itself (Howard and Buckley, 1985). Once activated, aerolysin binds with the glycophorin, a transmembrane protein at the surface of eucaryotic cells. Binding is followed by the production of transmembrane channels that leads to cell death (Buckley, 1992). Erythrocytes from different animal species have been shown to have different sensitivities to the lytic action of purified aerolysin (Bernheimer *et al.*, 1975).

A 15.5-kDa extracellular polypeptide of *A. hydrophila* has been shown to have acetylcholinesterase activity (AcChE-toxin) (Nieto *et al.*, 1991). The toxin was not cytolytic nor did it produce gross pathology, but had a minimum lethal dose of 0.05 μg/g when injected into fish. The AcChE-toxin was secreted extracellularly as a 45-kDa pro-toxin (Pérez *et al.*, 2002) and was split by other extracellular elements into lower molecular weight fragments, resulting in the production of a highly reactive 15-kDa polypeptide (Rodriguez *et al.*, 1993a). The toxin was detected *in vivo* in brain homogenates of morbid fish, suggesting that it exerted a critical effect on the central nervous system (Rodriguez *et al.*, 1993b).

Like many other bacteria, members of the genus *Aeromonas* have been shown to inject anti-host virulence determinants into the hosts via a type III secretion system (TTSS). Degenerate primers based on *lcrD* family genes (present in all TTSS) allowed identification of the TTSS gene cluster in *A. hydrophila* AH-1 (Yu *et al.*, 2004). By inactivating two TTSS genes (*aopB* and *aopD*), these investigators noted that the mutated bacteria were phagocytized readily, had decreased cytotoxicity for carp epithelial cells and were less virulent in blue gourami (*Trichogaster trichopterus*) than the isogenic wild strain. The DNA sequence of the aeromonad TTSS in AH-1 consisted of 35 genes, which was similar to *P. aeruginosa*. The TTSS was evident in clinical and environmental strains of other aeromonad species, including *A. hydrophila*, *A. veronii* and *A. caviae* (Vilches *et al.*, 2004).

Results of a recent molecular analysis (Zhang *et al.*, 2000) further underscore the genetic differences that exist between virulent and avirulent isolates and tend to confirm that virulence depends on a multiplicity of factors. Their results showed that 22 DNA fragments were present in most virulent strains and that these genes encoded for five of the known virulence factors of *A. hydrophila*, including haemolysin (hlyA), protease (oligopeptidase A), outer-membrane protein (OMP), multidrug-resistance protein and histone-like protein (HU-2). These same fragments were mostly absent in the avirulent isolates that were examined.

Similar to the motile aeromonads, clinical signs of furunculosis are produced readily

in fish injected with extracellular products produced during the growth of *A. salmonicida* (Ellis *et al.*, 1981) and an extensive body of research also exists on mechanisms of virulence associated with this pathogen. Although it is phenotypically difficult to predict virulence *in vitro* based on the presence or absence of an individual bacterial component (Olivier, 1990), the synergistic effect of different elements is indeed significant to the cumulative expression of furunculosis *in vivo*. The nature of virulence in *A. salmonicida* is indeed complex.

Similar to the S-layer of motile aeromonads, Udey (1978) showed that virulent *A. salmonicida* possessed an additional layer (A-layer) associated with the external surface of the membrane of the cell wall. The inability of *A. salmonicida* to produce A-protein results in its loss of virulence, whereas the virulence of *A. hydrophila* is not affected as severely by that bacterium's inability to produce an S-layer (Noonan and Trust, 1997). Evenberg *et al.* (1981) determined that the A-layer consisted of a major additional cell envelope protein (ACE) that was immunologically similar among virulent isolates. The protein was water-insoluble, hydrophobic and similar to the K88 adhesive fimbriae of enteropathogenic *E. coli*. It had a molecular weight of 49 kDa that was constructed in a tetragonal array beyond the bacterial cell wall (Kay *et al.*, 1981) and was present in both typical and atypical strains of the pathogen (Hamilton *et al.*, 1981). Kay and Trust (1991) demonstrated that the tetragonal array was tethered to the cell surface via the O-antigen side chains of bacterial lipopolysaccharide (LPS), which passed through the A-protein and were exposed on the cell surface. The chains were homogeneous in length and sugar composition and they were antigenically cross-reactive among typical and atypical isolates (Chart *et al.*, 1984). Lund and Mikkelsen (2004) determined that the A-layer proteins from *A. salmonicida* subsp. *salmonicida* were similar, but there was amino acid variability among those from atypical isolates that accounted for some antigenic diversity among atypical strains. The *O*-polysaccharide was a branched polymer composed of repeating trisaccharide units of L-rhamnose, D-glucose, 2-acetamido-2-deoxy-D-mannose, and an *O*-acetyl group (Wang *et al.*, 2005). Using capillary electrophoresis-mass spectrometry, Wang *et al.* (2007) further found three *O*-polysaccharide structural types (designated A, B and C). Most *A. salmonicida* subsp. *salmonicida* were Type A and produced a complete *O*-polysaccharide chain. The *O*-polysaccharide chain among atypical isolates from non-salmonids was incomplete and consisted of the polysaccharide backbone with or without an *O*-acetyl group substitution. Serological cross-reactivity existed between Types B and C, but not between Type A and non-Type A structural groups, indicative of two serogroups. Wang *et al.* (2006) also confirmed that the core oligosaccharide was structurally conserved.

The entire A-protein array blocks bacteriophage receptors on the outer membrane of *A. salmonicida* and provides a chemically refractive impermeant barrier (Trust *et al.*, 1981) that buffers the underlying outer membrane and its associated proteins from chemical modification by host defences including elements of immune function and complement lysis (Kay and Trust, 1991). Garduno *et al.* (2000) also showed that the A-protein promoted adherence and survival within macrophages. The A-protein could be reconstituted on to *A. salmonicida* that contained LPS and the presence of calcium enhanced its reattachment to cells (Garduno *et al.*, 1995). The tetragonal organization was affected by mutation of the *AsoA* gene, located 7 kb downstream of the *vapA* gene. Such mutation caused disorganization of the cell surface containing both A-layer and LPS (Noonan and Trust, 1995a). The resultant mutant (A449-TM1) could not secrete the A-protein through its outer cell membrane that caused its accumulation within the periplasm (Noonan and Trust, 1995b). The *vapA* gene sequences from typical *A. salmonicida* subsp. *salmonicida* were identical, whereas those from atypical strains varied, and that variability accounted for serological differences (Lund *et al.*, 2002; Lund and Mikkelsen, 2004).

When virulent and avirulent *A. salmonicida* were grown within intraperitoneal

implants in rainbow trout, novel antigens were detected that, except for LPS, were destroyed by proteinase K treatments (Thornton *et al.*, 1993). The LPS induced *in vivo* was antigenically distinct from that extracted from *in vitro* cultures. Furthermore, conditions of iron limitation and anaerobiosis did not induce *in vitro* expression of most of these antigens. Electron microscopy detected a putative capsule on *in vivo*-grown cells, which increased resistance to bacteriolysis and phagocytosis (Garduno *et al.*, 1993b). The authors also noted that the capsule shielded the underlying, regular surface array from immunogold labelling with a primary antibody to the A-protein. When grown in glucose-rich medium, *A. salmonicida* could produce capsular material, but these cells were functionally different from capsulated counterparts grown *in vivo* (Garduno and Kay, 1995). The *in vitro* cultured cells were sensitive to lysis by trout serum, whereas those grown *in vivo* were resistant and more adherent to trout macrophages. The presence of a capsule-like material has also been demonstrated on the surface of *A. hydrophila* cells grown *in vivo* (Mateos and Paniagua, 1995). The ability of *A. salmonicida* to invade fish cell lines was significantly higher when this capsular polysaccharide was present. Consequently, Merino *et al.* (1996) suggested that the capsule enhanced intracellular invasion. Merino *et al.* (1997) also found that serum-resistant strains of *A. salmonicida* activated complement but degraded C3b, which inhibited lysis by the C5b-9 complex. When grown under non-capsulating conditions, lipopolysaccharide rough mutants of *A. salmonicida* bound C3b, which alternatively led to production and lysis by the C5b-9 complex, as described above. When grown under capsulating conditions, however, these same strains bound less C3b and became more resistant to complement lysis than their non-capsulated counterparts. Even though *A. salmonicida* does not have visible pili, its genome contains genes for three type IV pilus systems: (i) Tap, similar to the *P. aeruginosa* Pil system; (ii) Flp, similar to the *Actinobacillus actinomycetemcomitans* Flp pilus; and (iii) a mannose-sensitive haemagglutinin pilus similar to *Vibrio cholerae* that was non-functional (Boyd *et al.*, 2008). In *A. salmonicida*, the Tap pili were polar and the Flp pili were peritrichous. Immersion challenges with a Tap-deficient mutant were less virulent than those with the wild-type strain, whereas the levels of morbidity were similar by intraperitoneal injection, suggesting an important attachment role for the Tap pili during contagion. Masada *et al.* (2002) also found that the Tap pili were immunogenic. Fish challenged with a wild-type pili-bearing strain were slightly more resistant to rechallenge with the wild-type strain than those that were challenged initially with a pili-deficient mutant.

When injected into fish in its purified form, bacterial endotoxin or LPS that is produced by *A. salmonicida* is not pathogenic (Wedemeyer *et al.*, 1969; Paterson and Fryer, 1974). In rainbow trout, Simko *et al.* (1999) demonstrated that LPS did not affect plasma proteins or exhibit acute phase changes induced by LPS inflammation in mammals. Within the extracellular products produced by *A. salmonicida*, LPS may aggregate with bacterial glycerophospholipid cholesterol acyltransferase (GCAT; molecular weight = 25 kDa). Lee and Ellis (1990) indicated that the GCAT/LPS complex (2000 kDa) was haemolytic, leucocytolytic, cytotoxic and lethal for Atlantic salmon when injected at a concentration of 0.045 μg protein/g of body weight. The LPS did not affect the enzymatic activity of free GCAT with egg yolk or phosphatidylcholine (lecithin). When complexed with LPS, GCAT was more heat stable, while haemolytic activity and lethal toxicity were about eightfold higher in the complexed form.

Injection of purified serine protease (AspA) from *A. salmonicida* into the dorsal aorta induced consumptive coagulopathy in Atlantic salmon (Salte *et al.*, 1993). Their results further suggested that the therapeutic effects of antithrombin injections were directed against generated thrombin and activated coagulation factor X, whereas those of alpha2-macroglobulin inactivated the protease directly. Eggset *et al.* (1994) deduced that about 10% of haemolytic

activity was attributed to the complex of LPS and GCAT, whereas more than 50% of that activity was a function of the free, 26-kDa GCAT serine protease. In their study, a transposon-produced mutant did not contain detectable amounts of the 26 kD molecule and had reduced haemolytic activity. Nerland (1996) sequenced the GCAT gene and found 92.9 and 93.7% homologies for the nucleotide sequence and deduced amino acid sequence, respectively, with the corresponding gene from *A. hydrophila*. Assessed by either injection or cohabitation challenges with Atlantic salmon, the virulence of neither GCAT-deficient nor serine protease AspA-deficient mutants of *A. salmonicida* was significantly different from that of their respective wild-type strains (Vipond *et al.*, 1998). Although these toxins enhanced pathogenicity, neither appeared to be a prerequisite for virulence.

Earlier studies indicated that, as a consequence of septicaemia, bacterial utilization of the host's blood sugar induced hypoglycaemia and subsequent death (Field *et al.*, 1944). At that time, very little was known about the potent action of the bacterial exotoxins produced by *A. salmonicida* until Griffin (1954) suggested that this bacterium produced a leucocidin that was responsible for the severe leucopenia observed histopathologically in initial stages of lesion development. Klontz *et al.* (1966) showed that this suppression of the inflammatory response could be produced by injecting trout with cell-free extracts from *A. salmonicida*. That work would initiate additional investigations of virulence associated with the exotoxic substances produced by *A. salmonicida* that continue to this day.

Fuller *et al.* (1977) extracted the leucocytolytic factor from the extracellular products, which not only was cytotoxic for leucocytes but synergistically enhanced bacterial virulence when injected in concert with viable cells. A protease was also extracted from the extracellular material (Shieh and MacLean, 1975) that induced the development of furuncle-like lesions in fish (Sakai, 1977). Cipriano *et al.* (1981) determined that the extracellular material was actually a composite of substances; one was analogous to the leucocytolytic factor described by Fuller *et al.* (1977), another was proteolytic and induced lesion development similar to that reported by Sakai (1977), and yet others expressed cytotoxic activity to rainbow trout gonad cells (RTG-2) or were haemolytic for trout erythrocytes and lethal to brook trout (*S. fontinalis*), regardless of the virulence of the strain from which they were extracted.

Shieh (1985) found that the proteolytic activity inherent among virulent isolates was quantitatively more toxic for Atlantic salmon than that isolated from avirulent isolates. Production of a protease-deficient mutant which lost virulence but maintained autoagglutinative, serum-resistant, adhesive, haemolytic and leucocytolytic activities provided further indication that extracellular protease was an important element of bacterial pathogenicity (Sakai, 1985). Based on chymotryptic properties, inhibition by diisopropylfluorophosphate (DFP) and tosylphenylalanine-chloromethyl ketone (TPCK) and hydrolysed *N*-benzoyl-L-tyrosine ethyl ester (BTEE), Tajima *et al.* (1984) characterized a 71-kDa heat labile alkaline serine protease from *A. salmonicida*. Proteolytic activity was stable from pH 5.0 to 10.0, but maximum activity was observed at pH 9.4 (50°C). Price *et al.* (1989) showed that strains of *A. salmonicida* actually produced a serine protease of molecular weight similar to that described by Tajima, which was active against casein and gelatin, and a 20-kDa protease, which was only active against gelatin. Because no difference was observed in the toxicity of extracellular products produced by caseinase-deficient mutant and its isogenic wild type, Drinan *et al.* (1989) inferred that the caseinase enzyme had no major role in the pathogenesis of *A. salmonicida* infections. Gudmundsdottir *et al.* (1990) described a 20-kDa protease from *A. salmonicida* subsp. *achromogenes* that was lethal for Atlantic salmon and characterized as a metalloprotease because of its inhibition by EDTA. A 35-kDa metalloprotease was also isolated from *A. salmonicida* subsp. *salmonicida* with optimum activity at pH 7.5 (40°C) against gelatin and azocoll but not against casein (Arnesen *et al.*, 1995).

The extracellular metallocaseinase, termed AsaP1, was linked with lethal toxicity and the development of furuncle-like lesions (Gunnlaugsdottir and Gudmundsdottir, 1997) and destroyed collagen in fish tissues (Prost, 2001).

An ADP-ribosylating toxin named *A. salmonicida* exoenzyme T (AexT) has also been identified and it is similar to the ExoS and ExoT exotoxins from *P. aeruginosa* and the YopE cytotoxin of *Yersinia* spp. (Braun *et al.*, 2002). Expression of the toxin required actual contact with RTG-2 cells, at which time AexT protein was either intracellular or tightly cell-associated and the cells underwent significant morphological change. In the absence of RTG-2 cells, AexT was not expressed.

Most typical and atypical isolates of *A. salmonicida* can grow under conditions of iron restriction, as evidenced by *in vitro* multiplication in the presence of iron chelators. Neelam *et al.* (1993) found that ethylenediamine dihydroxyphenylacetic acid (EDDA) caused slight increases in growth, whereas 2,2′-dipyridyl (Dipy) and 8-hydroxyquinoline (8HQ) reduced cell growth by 50 and 90%, respectively. They also noted that growth in the presence of chelating agents favoured expression of the 70-kDa major protease, as well as a proportional increase in 70- to 90-kDa proteins within the extracellular products. Ebanks *et al.* (2004) identified a 73-kDa colicin receptor, a 76-kDa haem receptor and an 85-kDa ferric siderophore receptor expressed as outer membrane proteins under iron-depleted conditions. Siderophore production was detected only among typical strains of *A. salmonicida* (Hirst *et al.*, 1991); consequently, it was inferred that atypical and typical isolates regulated iron uptake differently. Among typical strains, utilization was siderophore-dependent and transferrin was digested by the 70-kDa serine protease (Hirst and Ellis, 1996). The presence of the catechol siderophore and major iron-regulated outer membrane proteins (IROMPs) was consistent and homogeneous within *A. salmonicida* subsp. *salmonicida* (Fernandez *et al.*, 1998). Consequently, it would appear that the siderophore-independent utilization of transferrin within atypical isolates is a function of proteolytic digestion by the metalloprotease. Six genes (*asbG*, *asbF*, *asbD*, *asbC*, *asbB* and *asbI*) that encode siderophore proteins in other bacteria have been identified in *A. salmonicida* (Najimi *et al.*, 2008). Mohsen *et al.* (2008) demonstrated that the *asbD* gene (encoding a multidomain non-ribosomal peptide synthetase), the *asbG* gene (encoding a histidine decarboxylase) and the *asbC* gene (encoding a predicted histamine monooxygenase) were necessary to express the catechol siderophore that was essential for the growth of *A. salmonicida* under conditions of iron limitation.

Control, Treatment and Epizootiology

Effective management is the best approach to avoid infections and subsequent epizootics caused by all members of the genus *Aeromonas*. In water-reuse hatcheries, both ozonation (Colberg and Lingg, 1978) and filtration combined with ultraviolet irradiation (Bullock and Stuckey, 1977) effectively eliminate the threat of *A. hydrophila*. Colberg and Lingg (1978) showed that a specific microbial oxygen demand was exerted during batch ozonation that caused a greater than 99% mortality of bacteria within a 60 s contact during continuous flow exposure at 0.1–1.0 mg ozone/l. Calesante *et al.* (1981) eliminated recurrent motile aeromonad mortality among muskellunge (*Esox masquinongy*) fry by ultraviolet irradiation of lake and well water during egg incubation and yolk absorption.

Motile aeromonad septicaemias generally are mediated by stress. Elevated water temperature (Esch and Hazen, 1980), a decrease in dissolved oxygen concentration, or increases in ammonia and carbon dioxide concentrations have been shown to promote stress in fish and trigger motile aeromonad infections (Walters and Plumb, 1980). The monitoring of environmental variables can therefore enable one to forecast stressful situations and possibly avoid problems before they arise. Pond fish should not be handled but transferred only after the water

temperature is high enough for fish to be active and feeding normally (Rychlicki and Zarnecki, 1957). Mortalities were reduced dramatically (80–90%) when fish, at the time of spring transfer, were injected intraperitoneally with 10–20 mg of chloromycetin or by dissolving 10–20 mg chloromycetin in water/kg of fish (5–10 mg/lb). In the USA, however, this drug is not registered for use on food fish nor is it legal to treat fish prophylactically.

It is prudent that managers should avoid introducing fish into their hatcheries that have been infected recently. Shipments of new eggs should be surface disinfected to prevent contamination of facilities and stocks. Wright and Snow (1975) found that either acriflavine (500–700 mg/l for 15 min) or iodine as Betadine (100–150 mg/l iodine for 15 min) disinfected eggs of largemouth bass (*M. salmoides*) successfully, but neither Roccal nor formalin was effective. When warmwater fish are held in tanks or hauled in trucks or plastic bags, the value of adding disinfectants or antibiotics should be examined. The most promising compounds include oxytetracycline, chlortetracycline and a mixture of penicillin and streptomycin added to water at a rate of 10–15 mg/l. Again, it is important to note that the use and administration of such prophylaxis must be in compliance with local regulations.

Because furunculosis infects such a wide variety of hosts in a diversity of habitats, the bacterium is managed predominantly as a geographically ubiquitous but obligate pathogen. The pandemic distribution and prevalence of this bacterium, however, should never diminish the gravity of its pathogenic ramifications in the minds of fish culturists and resource managers. Reference to the factoral interplay of host, pathogen and environment is used so often that it becomes an abstract conceptualization of the disease process. Avoidance of *A. salmonicida* within populations of fish is critical to the prevention of furunculosis. Jarp *et al*. (1993) have found that the migration of anadromous fish into hatchery water supplies, a dense concentration of other infected fish culture stations proximate to uncontaminated stations and sharing personnel or equipment between stations are among the most significant risks associated with the introduction of furunculosis. Managers have some degree of control over each of these parameters. Ultraviolet irradiation (Bullock and Stuckey, 1977) or ozonation (Wedemeyer and Nelson, 1977; Colberg and Lingg, 1978) of incoming culture water, fallowing of net-pen sites and education of personnel can alleviate significant sources of potential contamination and prevent disease. Regular monitoring programmes that detect *A. salmonicida* in the water supply and provide early non-lethal detection on mucus can be coupled with topical disinfection or antibiotic regimens that either preclude or minimize infection (Cipriano, 1997). Furthermore, only fertilized eggs or stocks of fish that had been certified to be free of *A. salmonicida* infection should be transferred between facilities attempting to maintain a specific pathogen-free status. In practice, conduct of the stress-induced furunculosis assay and regulatory confinement of infected smolts have reduced the number of furunculosis outbreaks associated with early marine culture (Smith, 1991; Olivier, 1992).

Eyed eggs from sources not certified to be specific pathogen free should be disinfected on arrival and must be isolated from subsequent contact with other eggs or with contaminated packing material and containers. Povidone iodine has become the compound of choice for disinfecting trout and salmon eggs (McFadden, 1969), because it possesses greater bactericidal activity than merthiolates (Gee and Sarles, 1942) or acriflavine (Blake, 1930; Atkinson, 1932). The compound is available commercially as an iodophor (e.g. Betadine and Wescodyne) that generally contains approximately 1.0–1.6% active iodine in inert organic solvents (McFadden, 1969; Amend, 1974). Although Ross and Smith (1972) extended the effective use of povidone iodine against a number of bacterial fish pathogens including *A. salmonicida*, they also noted that disinfection was incomplete when 25 mg/l of iodophor was used for 15–120 min with certain strains of this pathogen. McCarthy (1977b) also found that a 10-min treatment

of artificially infected green or eyed eggs with 50 or l00 mg/l of active iodine (as Wescodyne) would not kill all *A. salmonicida* cells, but that a 30-min exposure to 1000 mg/l acriflavine was completely efficacious. Consequently, Piper *et al.* (1982) recommended that salmonid eggs should be disinfected in a solution containing 100 mg of active iodine/l for 10 min, but the bactericidal activity of iodophor would still be influenced by the concentration of *A. salmonicida* present and the degree of organic contamination in the disinfectant solution (Sako *et al.*, 1988). US Fish and Wildlife Service (USFWS) regulations recommend that eggs should be disinfected by submersion for 30 min in 50 mg iodine/l immediately after fertilization. If eggs are then shipped to another facility for incubation, policy requires that those eggs undergo a secondary disinfection in 100 mg iodine/l for 10 min (USFWS, 1995). Disinfection should be conducted in 10–15°C pathogen-free water at a pH of 7.0 (6–8). Soft water in which the normal acidity of iodophors could reduce effectiveness would be alkalized by adding 0.5 g sodium bicarbonate/l of water. The eggs should be rinsed immediately after treatment, unless placed into a flowing-water incubator within a few minutes after disinfection.

Immunization

Motile aeromonads are one of the most taxonomically and antigenically diverse groups of bacteria pathogenic to fish. The extent of antigenic diversity inherent within this group is especially expressed within H and O somatic antigens. Ewing *et al.* (1961) described 12 O-antigen groups and 9 H-antigen groups. Each group was further divided into a number of additional serotypes. Chodyniecki (1965) also found a high degree of antigenic diversity among strains of motile aeromonads obtained from the same population of fish, and even from different organs of the same fish. Initially, monovalent bacterins were prepared against *A. hydrophila*, but these vaccines only provided acceptable levels of protection against challenge with a homologous bacterium. Fish were not immune to infection by heterologous strains of *A. hydrophila* (Post, 1966; Schaperclaus, 1967). Although some strains of motile aeromonads have common somatic antigens (Rao and Foster, 1977; Lallier *et al.*, 1981), it has been demonstrated consistently that a monovalent antiserum could agglutinate only a small percentage of the total isolates examined. Kingma (1978) produced seven rabbit antisera to heat-stable antigens of seven isolates. Collectively, these antisera agglutinated only 19.5% of the total number of motile aeromonad isolates studied. Despite the tremendous degree of serological diversity, Mittal *et al.* (1980) found that strains of *A. hydrophila,* which were highly virulent for rainbow trout, shared a common O-antigen. Furthermore, these bacteria did not agglutinate in acriflavine, settled after boiling and resisted the bactericidal activity of normal mammalian sera. In contrast, low-virulence strains of *A. sobria* did not share the common O-antigen; these bacteria settled after boiling and were lysed by normal mammalian sera. Kozinska and Antychowicz (2001) found that immunization with monovalent bacterins against either *A. hydrophila* or *A. sobria* conferred only partial protection when the heterologous species was used in challenges.

Janda *et al.* (1996) found that the motile aeromonads were serologically heterogeneous and that most of the type or reference strains for each group were not serologically representative of the genomospecies at large. Mesophilic aeromonads, including *A. hydrophila* and *A. caviae*, have been divided into 44 serogroups based on O-antigen typing (Sakazaki and Shimada, 1984). Using this scheme, Thomas *et al.* (1990) successfully typed between 14 and 35% of the isolates that they examined from clinical and environmental specimens in the UK, Australia, Brazil, Peru and the USA. Also, another 52 new provisional O-antigen serogroups were identified, which increased typability to 76% for *A. hydrophila* and 63% for both *A. caviae* and *A. sobria*. By O-antigen characterization, LeBlanc *et al.*

(1981) could only type 39 of the 195 strains of *A. hydrophila* and *A. sobria* that had been isolated from fish. Dooley *et al.* (1985) found that LPS of *A. hydrophila*, obtained from isolates that were virulent for fish, had *O*-polysaccharide chains of homogeneous chain length with three epitopes on the polysaccharide moiety. One epitope was serogroup specific and was not present on the heterogeneous chain length *O*-polysaccharides of non-autoaggregating strains of *A. hydrophila*. Another epitope was conformation dependent and cross-reacted with an epitope on the homogeneous chain length *O*-polysaccharides of *A. salmonicida* lipopolysaccharide. The third epitope recognized by a monoclonal antibody involved the *O*-polysaccharide–core oligosaccharide glycosidic linkage of *A. hydrophila* and *A. salmonicida*.

Furthermore, Kostrzynska *et al.* (1992) demonstrated the S-layer proteins of the mesophilic aeromonads were antigenically diverse. Although surface array proteins were related structurally, they differed in primary sequence. Slide agglutination results indicated that most of the motile aeromonads (72%) virulent for fish fell within serotypes O3, O6, O11 and O19, but it should be noted that these groups also contained non-pathogenic strains as well (Santos *et al.*, 1996). Not only was there heterogeneity in LPS, cell envelop proteins and ECP among these different serogroups, but also there was significant diversity within each group. Esteve *et al.* (1994) found that most isolates of *A. hydrophila* isolated from epizootics in eels belonged to the O19 serogroup and consequently suggested that certain O-serotypes might serve as epidemiological markers.

Formalin-inactivated vaccines against motile aeromonads were superior to heat-killed preparations (Kozinska and Antychowicz, 2000), especially when the bacterins were injected with an adjuvant (Anbarasu *et al.*, 1998). Thune and Plumb (1982) found that both sac fry and swim-up fry vaccinated by immersion in sonicated polyvalent bacterin were protected against challenge with homologous bacteria, indicating an early onset of immunocompetence in channel catfish. This study is important because *A. hydrophila* causes a severe problem in channel catfish in spring and early summer, when fry are abundant. The early onset of immunocompetence in channel catfish fry indicates that immunization, if effective, can reduce outbreaks of *A. hydrophila* infection. Regardless of whether whole cells, freeze-thawed cells or cell sonicates were used, Thune and Plumb (1982) further indicated that injection was superior to either immersion or spray vaccination for developing humoral antibodies. However, cell sonicates evoked the best antibody response. Sonication would disrupt the cell and allow better processing of certain somatic antigens (e.g. bacterial LPS). Consequently, Baba *et al.* (1988) were able to evoke better protection against *A. hydrophila* in carp that were vaccinated with crude LPS rather than whole-cell, formalin-killed vaccine. Immersion of fish in the LPS vaccine for 2 h at 25°C was more efficacious and less stressful than injection, but vaccination with crude LPS did not invoke an observed humoral immune response, as measured using bacterial agglutination, passive haemagglutination and agar gel diffusion tests.

Majumdar *et al.* (2007a,b) used a live strain of *A. hydrophila* AO1, which was cured of its 21 kb virulence plasmid and became non-virulent in Indian catfish (*C. batrachus*). Following injection into Indian catfish, the plasmid-less strain spread to the kidney and spleen, but not in the same numbers as occurred with the wild-type virulent culture. Indeed, the non-virulent culture was cleared eventually from the recipient fish. However, the T-cell response was stimulated, serum antibodies were produced and vaccinated fish survived challenge with a virulent *A. hydrophila* and other *Aeromonas* spp. (Majumdar *et al.*, 2007b). The value of live attenuated vaccines for *A. hydrophila* was reinforced by Liu and Bi (2007), who used a transposon Tn916 generated mutant of *A. hydrophila* J-1, which was defective in its ability to produce extracellular enzymes, to vaccinate swordtail (*Xiphophorus helleri*). The result after challenge was 77% survival of the vaccinates which received

10^7 cells/fish compared with only 37% survival of the controls.

Schachte (1978) also found that the delivery of vaccine by injection or immersion or *per os* stimulated differential kinetics of antibody production. However, no significant protection against natural exposure to heterologous *A. hydrophila* was afforded to channel catfish vaccinated by any of the methods. To improve the efficacy afforded by an oral delivery, several studies have examined the development of biofilm vaccines in which cells of *A. hydrophila* are grown and harvested on chitin flakes and then heat inactivated at 90°C for 40 min. The biofilm vaccine was shown to elicit better humoral and protective responses than cellular bacterin (Azad *et al.*, 1999) and it was postulated that the glycocalyx of the biofilm might enhance immunogenicity by protecting the antigen from destruction in the gut (Azad *et al.*, 2000). Best humoral antibody titres and the highest level of protection were conferred by oral vaccination of carp fed the biofilm vaccine containing 10^{13} cfu/g of fish/day for a minimum of 15 days (Azad *et al.*, 1999). In biofilm preparations, Asha *et al.* (2004) found that S-layer proteins were lost but the LPS produced an additional high molecular weight band that possibly improved the protective response. Choi and Oh (2007) also showed some degree of protection from mortality and a reduction in skin lesions in carp that were orally immunized with liposome-entrapped preparations of *A. hydrophila*.

Because of the antigenic diversity, additional vaccination strategies included the development of polyvalent vaccines, immunization against inactivated extracellular toxins (toxoids) and the development of vaccines consisting of cellular antigens plus toxoid. Liu (1961) noted that the biological activity of extracellular toxins from motile aeromonads was neutralized by a single antiserum prepared against an isolate of *A. liquefaciens* (synonym for *A. hydrophila*). He therefore concluded that the motile aeromonads shared extracellular antigens. Bullock *et al.* (1972) also indicated that aerogenic and anaerogenic strains of *A. hydrophila* possessed common extracellular antigens. Schaperclaus (1970) recognized that common carp vaccinated against *A. hydrophila* developed both agglutinating serum antibodies against cellular antigens and antitoxic activity against extracellular antigens. In a later study, he found that common carp vaccinated by intraperitoneal injection of bacteria produced more circulating antibodies than did carp vaccinated by the oral route (Schaperclaus, 1972). Furthermore, vaccination with soluble extracellular antigens was more efficacious and provided a wider base of protection against heterologous serotypes than did vaccination with whole-cell antigen. Shieh (1987) found that Atlantic salmon immunized by intramuscular injections of extracellular protease from *A. hydrophila* were protected from challenge with the homologous and some heterologous isolates of *A. hydrophila*. These studies were repeated with similar results when *A. sobria* extracellular protease was used as the immunogen and challenges were conducted with homologous and heterologous strains of the bacterium (Shieh, 1990). The efficacy of the type of bacterin preparation, however, is influenced by the route of administration. Soltani and Kalbassi (2001) found that Persian sturgeon (*Acipenser persicus*) were protected by immersion in heat-killed bacterins, but the level of protection was less for the heat-killed bacterin than it was for either LPS or ECP bacterins when these were injected intraperitoneally. Some cross-protection was afforded in European eels immunized by intraperitoneal injection with major outer membrane protein (MOMP) preparations, which were relatively conserved among different O-antigen serogroups (Dong *et al.*, 2005). Vazquez-Juareza *et al.* (2005) also indicated that encoding major outer membrane proteins (OMP) of *A. veronii* into DNA vaccines afforded some protection in spotted sand bass (*Paralabrax maculatofasciatus*) against a homologous challenge.

Attenuated vaccines have been developed using growth deficient (Leung *et al.*, 1997), *aroA* (Vivas *et al.*, 2004) and exoenzyme and plasmid-cured (Majumdar *et al.*, 2007a,b) mutants of *A. hydrophila*. Each of these vaccines was immunogenic and

conferred protection against experimental challenge. DNA vaccines encoding major outer membrane proteins of *A. sobria* have also produced partial protection in experimental trials. Shome and Shome (2005) found that attenuated vaccines were somewhat more efficacious than toxoid preparation, with better survival and antibody production among intraperitoneal versus immersion deliveries.

Vaccines of *A. salmonicida* emulsified in oil adjuvants and delivered by intraperitoneal injection provide long-lasting protection and their use continues to be promoted in commercial aquaculture (Lillehaug *et al.*, 1992; Ellis, 1997). Both aqueous and oil-adjuvanted vaccines are produced commercially and may induce immunity at water temperatures as low as 2°C. Eggset *et al.* (1997) noted that even though an antibody response for *A. salmonicida* was delayed or strongly suppressed at this temperature, protection was still satisfactory at 18 weeks after vaccination. After 18 weeks, protection was reduced severely in fish reared at 10°C and given the aqueous vaccine, but a similar reduction was not apparent in fish that were vaccinated with the oil-emulsion vaccine (Killie *et al.*, 1997). Although commercial furunculosis vaccines administered in mineral oil yield significantly higher protection than aqueous-based vaccines, the mineral oil adjuvant may produce abdominal adhesions near the site of injection (Midtlyng, 1993). If severe, these adhesions may decrease growth rate (Midtlyng, 1994), the severity of which is inconsistent between farms and may or may not be significant when compared with the final growth weight of non-vaccinated fish (Midtlyng and Lillehaug, 1998). The risk of developing adverse side effects can be reduced substantially by increasing the size of the fish when they are vaccinated (Berg *et al.*, 2007). Although vaccine-induced adhesions may develop in the ovaries of intraperitoneally vaccinated fish, egg production and viability are not affected adversely (Treasurer and Cox, 2008). Furthermore, combination of ECP antigens with the oil adjuvant, and not the adjuvant alone, appears to be responsible for induction of such strong inflammatory reactions (Mutoloki *et al.*, 2006). It must be remembered, however, that vaccination does not guarantee further expression of furunculosis within – or transmission of furunculosis from – covertly infected vaccinated carriers (Hiney, 1999). Both amoxicillin (Inglis *et al.*, 1996) and oxolinic acid (Ford *et al.*, 1998) treatments administered simultaneous to vaccination have effectively reduced the resurgence of latent infections during immunization. Lunden *et al.* (1998) cautioned that both oxytetracycline and oxolinic acid suppressed antibody production and circulating lymphocytes. They also noted that phagocytic activity was stimulated by oxolinic acid and suppressed by oxytetracycline.

Like similar vaccines prepared against the motile aeromonads, attenuated *AroA* mutants of *A. salmonicida* have also been shown to be safe and efficacious vaccines (Vaughan *et al.*, 1993). This finding has led to the production of a commercially prepared attenuated vaccine (Marsden *et al.*, 1996) based on the complete deletion of the *AroA* gene.

The serologic relatedness among strains of *A. salmonicida* (Popoff, 1969; Paterson *et al.*, 1980) suggests that immunization of fish against atypical forms of this pathogen is a realistic possibility. Atlantic salmon vaccinated with a commercial vaccine against *A. salmonicida* subsp. *salmonicida* or with this vaccine and another prepared against an autogenous strain of *A. salmonicida* subsp. *achromogenes* were equally protected against *A. salmonicida* subsp. *salmonicida* by cohabitation challenge. However, salmon vaccinated only with *A. salmonicida* subsp. *achromogenes* were not protected against classical furunculosis (Gudmondsdottir and Gudmondsdottir, 1997). Furthermore, Evenberg *et al.* (1988) showed only moderate to slight protection against an atypical variant of *A. salmonicida* causing Carp Erythrodermatitis, when carp were immunized with either cell envelop preparations, purified LPS, purified A-layer protein and even formalinized whole cell bacterins. When detoxified ECP was used as the immunogen, carp were protected against a subsequent lethal challenge. Gudmundsdottir *et al.* (1997)

found that a significant immune response was presented by Atlantic salmon against the A-protein of *A. salmonicida* subsp. *achromogenes.* There is some degree of variability in the surface A-layer protein among atypical isolates (Lund *et al.*, 2002; Lund and Mikkelsen, 2004), but no clear correlation between vaccine efficacy and A-protein group has been demonstrated (Lund *et al.*, 2008).

Chemotherapy

Oxytetracycline has been the drug of choice for treating motile aeromonad septicaemias in fish. In the USA, this drug is approved for use with pond fish, channel catfish and salmonids. It is administered for 10 days in the feed at a daily rate of 50–75 mg/kg of fish. Fish must be withdrawn from treatment for 21 days before they are stocked or eaten. This treatment sometimes produces dramatic results when it is administered for even 2 or 3 days, and is particularly effective when fish become infected after they have been handled, crowded or held under stress for short periods of time (Meyer, 1964; Meyer and Collar, 1964). Furanace, though not registered for use in the USA, is extremely effective against motile aeromonads if the affected fish are immersed for 5–10 min in water containing 1–2 mg/l furanace, or by maintaining fish for 1 week in water containing 0.1 mg/l drug. However, furanace can be toxic to fish if used improperly (Mitchell and Plumb, 1980). Chloramphenicol (chloromycetin) was used successfully to treat frogs with red leg disease by gastric intubation of 3–5 mg/100 g of frog for 5 days, twice daily. Chloromycetin, like oxytetracycline, is effective in treating fish when it is administered orally. However, its use is prohibited in food fish and discouraged in other fish because it is the drug of last resort in certain human diseases (e.g. typhoid fever). Indiscriminant use of chloramphenicol can result in drug resistance, and thus reduce the value of the antibiotic in human medicine. Against strains of motile aeromonads that show multiple drug resistance, piromidic acid administered orally has been shown experimentally to be more effective than either chloromycetin or oxytetracycline (Katae *et al.*, 1979). However, this drug is not registered for use on food fish in the USA. The potentiated sulfonamides trimethoprim-sulfadiazine (Liu and Liu, 1995) and trimethoprim-sulfamethoxazole (Ozturk *et al.*, 2007) have been used for the treatment of motile aeromonad septicaemias in eels and carp, respectively.

Furunculosis of salmonids was the first disease of fish to be treated with sulfonamides and nitrofurans (Gutsell, 1948). Although other drugs control this disease effectively (Herman, 1970), the US Food and Drug Administration enforces stringent requirements for drugs used on food animals in the USA, and only sulfamerazine (no longer registered), oxytetracycline and the potentiated sulfonamide Ro5-0037 or ROMET® (Bullock *et al.*, 1974) may be used. Although most sulfonamides are no longer used, sulfamerazine (American Cyanamid, Co.) has been approved to treat furunculosis in rainbow trout, brook trout and brown trout at a dose of 200 mg of drug/kg of fish/day (10 g/45.3 kg of fish weight) for 14 days. Treatment must terminate at least 3 weeks before fish are to be marketed or stocked. Sulfisoxazole (gantrisin) is preferred for treating brown trout, whose growth may be inhibited by sulfamerazine (Snieszko and Wood, 1955).

Oxytetracycline (Terramycin®, monoalkyl trimethyl ammonium, Pfizer, Inc.) is cleared for use in all species of salmonids, at the rate of 50–80 mg drug/kg of fish/day (2.5–3.75 g/45.3 kg of fish) for 10 days. Again, treatment must terminate at least 3 weeks before fish are released. Uhland and Higgins (2006) determined susceptible and non-susceptible minimum inhibitory concentration (MIC) breakpoints of *A. salmonicida* to range from $\leq 1\,\mu$g/ml and $>16\,\mu$g/ml for oxytetracycline (OTC) and $\leq 1\,\mu$g/ml and $>4\,\mu$g/ml for tetracycline (TET), respectively. The researchers equated these non-susceptible and susceptible breakpoint values on antibiotic discs to 15 mm and 26 mm zones of inhibition for OTC and 21 mm and 28 mm for TET, respectively. The potentiated sulfonamide, ROMET®

(Hoffman-LaRoche, Inc.), which consists of five parts sulfadimethoxine and one part ormetoprim, controlled furunculosis when fed at the rate of 50 mg/kg of fish/day (2.5 g/45.3 kg of fish) for 14 days (Bullock *et al.*, 1974) and has been cleared for use at the same dosage when administered for 5 days, with a 42-day withdrawal period. Resistance to both oxytetracycline (Adams *et al.*, 1998; McIntosh *et al.*, 2008) and ROMET (Starliper and Cooper, 1998; Schmidt *et al.*, 2001) is encoded by high molecular weight R-plasmids. Miller and Reimschuessel (2006) found that atypical *A salmonicida* isolates were generally more susceptible than their typical counterparts against oxytetracycline, ormetoprim-sulfadimethoxine and florfenicol, but less susceptible to oxolinic acid.

Due to increased resistance against tetracycline and the potentiated sulfonamides, certain quinolones were evaluated as replacement antibiotics. Oxolinic acid was among the first of the quinolones to be evaluated and has been widely used in aquaculture. Austin *et al.* (1983) originally reported successful control of furunculosis in brown trout and rainbow trout administered the drug orally at 10 mg/kg of body weight/day for 10 days. Bowser *et al.* (1990) found that treatments of Atlantic salmon with this regimen and treatments of hybrid brook trout (*S. fontinalis*×*S. namaycush*) with 5 mg of oxolinic acid/kg of fish extended for 10 days were ineffective. Nevertheless, use of oxolinic acid at 10 mg/kg of body weight/day for 10 days afforded protection (Verner-Jeffreys *et al.*, 2007).

As commonly occurs with the increased usage of any antibiotic, bacterial resistance to oxolinic acid has exacerbated efficacious control. Hastings and McKay (1987) reported on the failure of oxolinic acid to control outbreaks of furunculosis in Atlantic salmon and found that MICs of oxolinic acid for resistant strains ranged between 1.25 and 5.0 μg/ml, whereas those for sensitive strains were $\leq$0.02 μg/ml. Examining the nature of antibiotic resistance among isolates of *A. salmonicida* obtained from furunculosis outbreaks in Scotland between 1988 and 2000, Inglis *et al.* (1991a) found that 55% of the *A. salmonicida* strains were resistant to oxytetracycline and 37% were resistant to oxolinic acid. Barnes *et al.* (1991) reported that other fluoroquinolones (e.g. sarafloxacin and enrofloxacin) were more biologically active against *A. salmonicida* and resistant mutants to these antibiotics developed at a lower frequency than they did to either oxolinic acid or oxytetracyline. Bowser *et al.* (1990) found that treatment of hybrid brook trout (*S. fontinalis*×*S. namaycush*) with 10 mg of enrofloxacin/kg of body weight/day for 10 days was effective. Field trials in which lake trout (Hsu *et al.*, 1995) or Atlantic salmon (Stoffregen *et al.*, 1993) were treated with 10 mg of enrofloxacin/kg of body weight/day for 10 days were also effective. Injection of enrofloxacin at 5 mg active ingredient/kg of fish prevented the formation of characteristic furunculosis lesions caused by atypical isolates of *A. salmonicida* (Williams *et al.*, 1997), but did not reduce resultant mortality in Atlantic tomcod (*Microgadus tomcod*). Some control, however, was manifested by multiple injections. Another fluoroquinolone, flumequine, was shown to control *A. salmonicida* effectively when applied: (i) orally at 40 g/kg of food fed the rate of 1% body weight/day for 7–10 days (Michel *et al.*, 1980); (ii) as a bath treatment (50 ppm, pH 7.0 at 11°C); and (iii) by intraperitoneal injection at 30 mg/kg of fish (Scallan and Smith, 1985). Ellingsen *et al.* (2002) found that concentration of flumequine administered at 5 and 10 g/kg produced effective drug concentrations in the livers and kidneys of treated fish. Because fish treated daily at the higher dosage had reduced appetites, the authors preferred to treat at the 5 g/kg dose.

In the early 1990s, increased resistance to fluoroquinolones was a cause of concern among regulatory agencies because use was believed to increase the risk of enhancing drug-resistant zoonotic infections. After treatments, the drug persisted for approximately 5 days in sediments below net pens at concentrations ranging from 0.05 to 0.2 μg/g (Bjorklund *et al.*, 1991). Residues of oxolinic acid and oxolinic acid-resistant *A. salmonicida* were found in cultured and

wild fish and mussels near medicated fish farms (Samuelsen *et al.*, 1992). Consequently, further usage of this class of compound was banned in many nations.

The *β*-lactam antibiotic, amoxicillin, was also shown to control experimental furunculosis in Atlantic salmon parr when administered orally at 80 mg of active ingredient/kg of body weight (Inglis *et al.*, 1992). Efficacy, however, may depend on the subspecies to be treated. Barnes *et al.* (1994) noted that isolates of *A. salmonicida* subsp. *salmonicida* were susceptible and had MIC values that ranged from 0.30 to 1.50 mg/l, whereas *A. salmonicida* subsp. *achromogenes* isolates were resistant (MICs > 500 mg/l). The mechanism of enhanced resistance was associated with the production of a *β*-lactamase enzyme by the atypical isolates. Nordmo *et al.* (1998b) showed that florfenicol was slightly more effective than oxolinic acid and trimethoprim/sulfadiazine against *A. salmonicida* in Atlantic salmon smolts in seawater. Samuelsen *et al.* (1998) treated experimentally challenged Atlantic salmon effectively with 10 mg florfenicol/kg of fish daily for 10 days. In field trials, Atlantic salmon treated with florfenicol had greater specific pathogen-associated survival than those lots of fish that were fed oxolinic acid, flumequine or a combination of trimethoprim and sulfadiazine (Nordmo *et al.*, 1994). Florfenicol was palatable to fish and feeding the drug for 10 days at 100 mg/kg, or for more prolonged periods at 50 mg/kg and 10 mg/kg, did not induce any aberrant feeding behaviours or pathologies (Inglis *et al.*, 1991b). Florfenicol (AQUAFLOR®, Schering-Plough) was approved recently to control furunculosis in salmonids in the USA at 10 mg florfenicol/kg of body weight for 10 days, with a 12-day withdrawal period.

Probiotics

In terrestrial animal use, beneficial organisms used in probiotics have centred on the lactic acid-producing bacteria, i.e. putative lactobacilli. In aquaculture, a far wider range of organisms has been evaluated, not all of which have been live products or applied orally. After evaluating the beneficial effects of *P. fluorescens* against vibriosis in rainbow trout, Gram *et al.* (1999, 2001) tested its potential as a probiotic against furunculosis in Atlantic salmon. The probiont strain of *P. fluorescens* exerted strong antagonism against *A. salmonicida in vitro*, but it did not reduce furunculosis-related mortality. Ringø *et al.* (2006) determined that the gastrointestinal microflora of cod (*Gadus morhua*) was influenced by diet. Gram-positive *Brochothrix* spp. and *Carnobacterium* spp. dominated the guts of those fish offered fishmeal, whereas Gram-negative *Chryseobacterium* spp. and *Psychrobacter glacincola*, in addition to *Carnobacterium* spp., were most prevalent in fish fed soybean meal, and *Psychrobacter* spp. dominated the guts of cod offered bioprocessed soybean meal. In these studies, the carnobacteria were antagonistic to both *A. salmonicida* subsp. *salmonicida* and *V. anguillarum*. *C. maltaromaticum* (B26) and *C. divergens* (B33) at $>10^7$ cells/g of feed fed to rainbow trout persisted within the intestines of treated fish for 3 weeks, and treated fish were protected against challenges with either *A. salmonicida* or *Y. ruckeri* (Kim and Austin, 2006). Differential immune functions were stimulated by the two probionts. Trout fed B26 had increased phagocytic activity among head kidney macrophages. Those fed B33 developed increased levels of respiratory burst and serum lysozyme functions. Ringø *et al.* (2006) found that *C. divergens* was an effective probiotic, even though it failed to ameliorate damage to intestinal mucosal epithelia observed in *in vitro* studies where the bacterium was co-cultured with either *A. salmonicida* or *V. anguillarum*. Strains of *Bacillus* sp. (JB-1) and *A. sobria* (GC2) that were isolated from the digestive tracts of rainbow trout and ghost carp (*Cyprinus* sp.), respectively, effectively controlled mortality caused by *A. salmonicida*, *Lactococcus garvieae*, *Streptococcus iniae*, *V. anguillarum*, *V. ordalii* and *Y. ruckeri* among rainbow trout offered the probionts at 2×10^8 cells/g of feed for 14 days (Brunt *et al.*, 2007). Both probiotic strains produced siderophores and chitinase and stimulated lysozyme, phagocytic and

respiratory burst activities in the treated trout. *B. subtilis* AB1 protected rainbow trout fingerlings against challenge with motile *Aeromonas* sp. when fed as a viable, formalized and sonicated or cell-free preparation for 14 days at a dose equivalent to 10^7 cells/g of feed (Newaj-Fyzul *et al.*, 2007). Again, there was evidence of stimulation of the innate immune response, specifically enhanced respiratory burst activity, serum and gut lysozyme levels, peroxidase, phagocytic killing, anti-protease and increased lymphocyte populations compared with controls (Newaj-Fyzul *et al.*, 2007). In this study, it was apparent that viable bacterial cells were not absolutely required for protection against challenge.

In addition, strains of *Lactobacillus* are also widely used as probiotics (Tannock, 2005). Salinas *et al.* (2006) studied the *in vitro* effects of four heat-inactivated bacterial species on the cellular innate immune responses of the gilthead sea bream (*Sparus aurata*). They found that leucocyte peroxidase content was significantly higher after incubation with an isolate of *Shewanella* sp. (51M6). Head-kidney phagocytes actively phagocytized *L. delbrueckii* subsp. *lactis* and *B. subtilis*, while incubation of sea bream leucocytes with 51M6, *L. delbrueckii* subsp. *lactis* and *B. subtilis* increased respiratory burst activities. Kim *et al.* (2007) determined that cell-free supernatants of *L. amylovorus* S6 had antagonistic activity against the fish pathogens *A. salmonicida* subsp. *salmonicida* and *Edwardsiella tarda*, and this was equivalent to the inhibition afforded by antibiotics, including oxytetracycline. Balcazar *et al.* (2006) observed that although phagocytosis of heat-inactivated *A. salmonicida* was enhanced among gut leucocytes of rainbow trout fed *L. lactis* subsp. *lactis*, *L. sakei* and *Leuconostoc mesenteroides*, phagocytosis of live *A. salmonicida* was enhanced only in fish offered *L. lactis* subsp. *lactis*. Oral administration of *L. lactis* subsp. *lactis* (CLFP 100), *L. mesenteroides* (CLFP 196) and *L. sakei* (CLFP 202) as probiotics for 2 weeks at 10^6 cfu/g of feed enhanced survival against experimental challenges of rainbow trout with *A. salmonicida*, ranging from 97.8 to 100% compared with 65.6% in untreated controls (Balcazar *et al.*, 2007b). Probiotic *L. lactis* (CLFP 101), *L. plantarum* (CLFP 238) and *L. fermentum* (CLFP 242) reduced adhesion of *A. hydrophila* and *A. salmonicida* to host intestinal mucus, but only the spent culture medium from *L. lactis* (CLFP 101) was antagonistic to these pathogens (Balcazar *et al.*, 2008). *L. delbrueckii* subsp. *lactis* persisted within the gastrointestinal tract of probiotic-treated Atlantic salmon, and pretreatment of salmon intestine with this probiont reduced *A. salmonicida* damage to the intestinal lining (Salinas *et al.*, 2008).

Apart from bacteria, live baker's yeast (*Saccharomyces cerevisiae*) has been evaluated to control *A. hydrophila* infection in Nile tilapia (*O. niloticus*) fry (Abdel-Tawwab *et al.*, 2008). Fish fed for 12 weeks with diets supplemented with 5.0 g yeast/kg of feed had low mortalities after intraperitoneal challenge. Also, there was evidence of growth promotion and, in particular, increased protein deposition (Abbel-Tawwab *et al.*, 2008).

Dietary supplements

Various dietary supplements have been evaluated to improve health in recipient animals. Vitamins A, C and E are useful in controlling *A. hydrophila* (Sobhana *et al.*, 2002). Furthermore, vitamin C stimulates humoral and cell-mediated immune response in fish vaccinated with *A. hydrophila* vaccines (Anbarasu and Chandran, 2001). ß-glucans have been associated with enhancing non-specific resistance to *A. hydrophila* infection (Selvaraj *et al.*, 2005; Kumari and Sahoo, 2006). Yeast RNA incorporated in diets at 0.4% (w/v) and fed for 60 days reduced mortalities caused by *A. hydrophila* in rohu (*L. rohita*) and enhanced the phagocyte respiratory burst activity (Choudhury *et al.*, 2005). Microbial levan was fed for 60 days to rohu carp, followed by challenge with *A. hydrophila* (Gupta *et al.*, 2008). An increase in haemoglobin, erythrocyte and leucocyte count, serum lysozyme and respiratory burst activities occurred with increasing amounts of dietary levan. The lowest

mortalities after challenge were recorded with the highest amount of levan, i.e. 1.25% (Gupta *et al.*, 2008). The benefits of medicinal plants have been the focus in many studies originating from Asia. Two Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) dosed at 0.1% in combination with 0.5% boron and fed for 4 weeks led to greatly reduced mortalities in Nile tilapia (*O. niloticus*) after challenge with *A. hydrophila*. Again, there was evidence of immunostimulation (Ardo *et al.*, 2008). In addition, the Indian medicinal herb, *Azardirachta indica*, demonstrated *in vitro* inhibitory activity against *A. hydrophila* (Harikrishnan and Balasundaram, 2005). The benefit of using 5 g of mango (*Magnifera indica*) kernel/kg of feed administered orally for 60 days to rohu carp fingerlings was highlighted by Sahu *et al.* (2007a), who reported almost complete survival (98% survival compared with 50% survival of the controls) after challenge with *A. hydrophila*. Similar to the other studies, there was evidence of immunostimulation, including increased serum bactericidal activity, lysozyme, superoxide anion production and serum protein and albumin levels compared with the controls. Garlic (*Allium sativum*), which was fed at 0.1–0.5 g/100 g of feed for 60 days, immunostimulated (lysozyme and serum bactericidal activities, serum protein and albumin levels and superoxide anion production) and protected rohu carp fingerlings against challenge (57% survival of the controls compared with 85% survival in the groups receiving garlic) with *A. hydrophila* (Sahu *et al.*, 2007b). Extracts of humus were administered orally to 5–10% to common carp (*C. carpio*) and led to protection against challenge with atypical *A. salmonicida* (Kodama *et al.*, 2007).

Selective breeding

Selective breeding programmes have been developed in salmonids to enhance experimental and field resistance to furunculosis. Brook trout at the Hackettstown (New Jersey) State Fish Hatchery were selected on the basis of growth, coloration and the ability to survive natural epizootics of furunculosis. Subsequent progeny were evaluated continually for acquired resistance to disease, and furunculosis mortality was reduced from 98 to 30.8%, after four successive generations of selection (Embody and Hayford, 1925). Resistant progeny grew faster than non-selected fish, which corresponded with a rise in the average number of eggs obtained per female at first and second spawns, but this was also accompanied by a gradual decrease in egg viability to the eyed-egg stage. Although the brook trout had acquired resistance to furunculosis, selection did not enhance resistance to Bacterial Gill Disease or infestations by *Gyrodactylus* and *Chilodonella* (Hayford and Embody, 1930). Probably the most successful selection study involves the development of furunculosis-resistant strains of trout by the New York State Department of Environmental Conservation (Rome, New York). Wolf (1953) collected 11 strains of brook trout and seven strains of brown trout (*S. trutta*) from the New England area that were inbred for a minimum of eight previous generations. The fish were constantly subjected to *A. salmonicida* via bath, horizontal and dietary challenges. Mortality within strains ranged from 36 to 66% for brook trout and from 5 to 99.7% for brown trout, and Wolf concluded that enough variation existed among the resistance of various lots to warrant further evaluation through selective breeding. Ehlinger (1964) continued these studies and cross-bred survivors between strains with intermediate or low mortality. Potential broodstock was further selected on the basis of size, coloration and egg yield. Resistance was monitored within strains as a function of eliminating susceptible breeders within a line and by experimental challenge on subsamples of the resultant progeny. During the next 10 years, one resistant strain of brook trout and one strain of brown trout were produced, which had practical application to the culture of salmonids throughout New York (Ehlinger, 1977). Acquired resistance to furunculosis of the Rome trout is widely accepted and these fish are currently used in

many hatchery programmes in the New England and the Mid-Atlantic regions (Kincaid *et al.*, 1997) for their survival and performance in recreational fisheries where furunculosis is enzootic (Hulbert, 1985).

Detailed genetic studies have shown that heritability of mortality among families of Atlantic salmon challenged by cohabitation with infected cohorts was high, $h^2=0.32+/-0.10$ and $0.48+/-0.17$, for the sire and dam components, respectively (Gjedrem *et al.*, 1991), suggesting that resistance to furunculosis could be improved effectively by selective breeding (Gjedrem and Gjoen, 1995). These observations were consistent with those of Bailey *et al.* (1993), who also indicated that heritabilty ($h^2=0.32+/-6$) in Atlantic salmon for resistance to furunculosis was sufficient to warrant further selection. Langefors *et al.* (2001) screened MHC class IIB genotypes and established exon 2 sequences encoding the major part of the peptide-binding region among families of Atlantic salmon that demonstrated either high resistance (HR) or low resistance (LR) to *A. salmonicida*. Three alleles were discerned from these studies, which showed some correlation with resistance to furunculosis. One allele, e, was significantly more prevalent in HR families than in LR families. Broodfish carrying the e allele had a 12-fold greater chance of being HR than cohorts without the e allele. A second allele, i, had a significantly higher frequency among those fish that either remained uninfected or survived experimental challenges than it did among dead individuals. A third allele, j, was prevalent in LR families and among individuals that died from infection.

Variable degrees of resistance have also been noted among non-salmonids in response to atypical isolates of *A. salmonicida* infections. Dubois-Darnaudpeys and Tuffery (1979) showed that tench (*Tinca tinca*) were more resistant to Carp Erythrodermatitis than the common carp, grass carp and silver carp, with degrees of resistance in descending order. Sovenyi *et al.* (1988) found that 3-month-old progeny of hybrid crosses between the common carp from Hungary and Japanese coloured carp had a body weight ($43.6+/-22.4$ g) and condition factor ($8.85+/-1.05$ g/cm^3) significantly higher than those of the common carp purebreds ($23.5+/-12.3$ g body weight; $8.53+/-0.62$ g/cm^3 condition factor). Furthermore, the hybrids sustained only half the mortality compared with the purebred carp after experimental challenge with an atypical *A. salmonicida*. Houghton *et al.* (1991) showed that a Polish strain of carp (inbred for six generations) was significantly more susceptible to Carp Erythrodermatitis than a Hungarian strain (inbred for five generations). Wiegertjes *et al.* (1993) further demonstrated that even within the same strain of fish, resistance to erythrodermatitis increased with age, as indicated by a shift from subacute (85% at 3 and 5 months) to chronic (40% at 10 months) mortality. Some evidence for genetic variations in the ability of rohu carp fingerlings to survive challenge with *A. hydrophila* has also been documented (Das *et al.*, 2008).

Bacteriophage

The use of bacteriophage therapy has been discussed as a possible means of disease control. However, as the host bacterium can develop resistance to previously lytic bacteriophage, the use in disease control is questionable. In one specific example involving furunculosis in Atlantic salmon, the use of bacteriophage did not reduce mortalities after challenge with *A. salmonicida* (Verner-Jeffreys *et al.*, 2007).

Conclusions

The aeromonads are among the best studied of all the fish/shellfish pathogens. Yet there are fundamental questions that remain unanswered. For example, what is the taxonomic status of the so-called atypical isolates of *A. salmonicida*? Should new subspecies be described or should the existing classification be scrapped with reference made only to the species, i.e *A. salmonicida*? Similarly, are all the historical reports describing motile aeromonas septicaemia as *A. hydrophila* or

its synonyms or is there more involvement of other *Aeromonas* spp. in disease processes? Developments in ecology and epizootiology have produced fascinating insights into fundamental biological questions, the result of which is the acceptance that motile aeromonads are common in the aquatic environment, whereas *A. salmonicida* is more restricted in its habitats. The role of NCBV cells is controversial – does it mean that the cells are truly dormant, damaged or senescent? Perhaps it means that culturing technology is not as adequate as envisaged, but a more fundamental question is why bacterial cells form visible growth in the first place. Surely, this is unnatural. However, the role of NCBV cells of *A. salmonicida* in fish health is certainly not resolved. Could these cells revert to virulence and under what conditions? A great deal of progress has been made regarding the understanding of pathogenicity and pathogenic mechanisms. Detailed knowledge exists about the relevance of S-layers and extra- and intracellular components. Of course, one of the overriding considerations is how disease may be controlled. The application of vaccine technology to fish diseases started with *A. salmonicida* in the 1940s and now commercial products based on iron-regulated outer membrane proteins (IROMPs) of typical strains are available. Motile aeromonads have attracted less interest in vaccine development. However, these pathogens have been the focus of probiotics, immunostimulants and medicinal plants, some of which stimulate immune memory. The use of genetically disease-resistant stock has already been considered for *A. salmonicida*. With the advent of the molecular age, this concept must receive further work. It is clear that aeromonads remain serious constraints to aquaculture, and this seems likely to continue for the foreseeable future.

Recommendations for Future Studies

1. The taxonomic status of motile aeromonads needs to be assessed carefully in terms of the possibility for the involvement of species other than *A. hydrophila* in the disease processes.
2. Improved diagnostic procedures are needed and should reflect advances in other areas of veterinary and human medicine. The current dominance of biochemical tests is likely to lead to misidentification.
3. More work is needed to explain definitely the natural habitat for *A. salmonicida* – is it really restricted to fish, or does it also occur elsewhere in the natural environment?
4. Work on pathogenicity mechanisms needs to be directed at the level of the gene and its expression.
5. Disease control mechanisms have advanced considerably over the past decade. However, new-generation vaccines and other approaches, e.g. specific chemically defined immunostimulants, are needed.

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13 Enteric Redmouth Disease (ERM) (*Yersinia ruckeri*)

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Introduction

Enteric redmouth (ERM) is an acute or chronic generalized bacterial septicaemia in fish caused by *Yersinia ruckeri*. The causative agent, a Gram-negative enteric bacterium, which was first isolated in the Hagerman Valley, Idaho, USA, in the early 1950s, was described fully by Ross *et al.* (1966) and defined as a new species, *Y. ruckeri,* in 1978 (Ewing *et al.*, 1978). The early terminology, redmouth, redthroat or bacterial septicaemia, used to describe the disease was derived from the gross clinical signs which, in many cases, were caused by mixed infections of Gram-negative organisms, particularly *Aeromonas liquefaciens*. In 1971, McDaniel proposed renaming the disease Hagerman Redmouth in a presentation to the US Trout Farmers Association, arguing the need to distinguish *Y. ruckeri* infections from other infections presenting similar clinical signs (McDaniel, 1971). In many cases where *Y. ruckeri* was isolated and almost certainly the causative agent, classical signs were absent. Standardization of the nomenclature as 'enteric redmouth' was recommended by the Fish Health Section of the American Fisheries Society in 1975 and this, with 'yersiniosis', remains the most acceptable name. The placement of the causative agent in the genus *Yersinia* (Ewing *et al.*, 1978) has also proved problematic, but this remains acceptable and is now strongly supported by molecular phylogeny of the 16S rRNA gene. Since its isolation in the Hagerman Valley, ERM either has spread or been recognized in most areas of the world where freshwater fish are cultured intensively in cold or temperate water. Losses of 10–15% over a growth cycle are common, and individual outbreaks may result in much higher mortalities. In small-scale, artificial challenge trials, where no intervention takes place, over 90% mortality may occur. It may be controlled to acceptable levels by good husbandry, particularly high water quality, and it generally responds well to antibiotics. The introduction of effective vaccines as early as 1970 and their use throughout many affected countries have reduced the impact of the disease substantially. Nevertheless, it presents a constant threat, requiring management procedures and treatments that erode the profitability of the farms where it is endemic. Recent emergence of novel strains of *Y. ruckeri* that are not controlled effectively by current vaccines has led to a plethora of new research into the pathogenesis and virulence of the causative agent using molecular techniques.

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Enteric Redmouth Disease

The early signs of *Y. ruckeri* infection in the acute phases of infection are typical of many other Gram-negative bacterial septicaemias where anorexia, darkening of the skin and lethargy almost always precede the disease, except in small fry, where deaths may occur without clinical signs (Kawula *et al.*, 1996). The reddening of the throat and mouth, caused by subcutaneous haemorrhaging and from which the disease received its name, is commonly, but not invariably, present. If the disease progresses without treatment, erosion of the jaw and palate may occur. Haemorrhage occurs on the body surface, at the gill tips, at the base of the fins and around the lateral line. Rarely, these may progress to ulcers. The vent area may also become inflamed, both externally and internally, at the distal end of the intestine. Exophthalmia has been reported in some cases in the later stages of infection, with haemorrhaging of the ocular cavity and iris (Fuhrmann *et al.*, 1983, 1984). This has been referred to as a separate condition in Australian trout (Llewellyn, 1980). Internally, there are congestion of the blood vessels throughout the peritoneum, and petechial haemorrhages, affecting liver, pancreas, swim-bladder, lateral muscles and adipose tissues associated with the pyloric caecae (Wobeser, 1973). The kidney and spleen may be swollen and there may be fluid in both the stomach and the intestine which has a yellowish, opaque, mucoid appearance (Busch, 1982). The spleen may increase up to threefold in size and this is associated with influx of bacteria, which increase in number over the first 3 days of infection in trout before declining (Raida and Buchmann, 2008a). Light microscopy shows bacteria in virtually all tissues, particularly the kidney, heart, liver and gills. Microscopic observation in the gill has been supported recently using *in vitro* expression analysis of a strain carrying a *yrp1:LacZ* fusion, in which high expression of the fusion protei was detected in both gill and intestines (Fernandez *et al.*, 2003). There may be severe necrosis of the haemopoietic tissues of the kidney. Wobeser (1973), Quentel and Aldrin (1986) and Lehmann *et al.* (1987) observed acute anaemia, with an average haematocrit as low as 23% and total serum-protein values of 2.8 g/100 ml. Miller (1983) attributed this to the effects of endotoxin on coagulation ultimately producing thrombosis in the capillaries and generalized haemorrhaging. Many potentially pathogenic Gram-negative organisms persist in small numbers in the intestine of apparently healthy fish and remain undetected in standard health checks. Viable bacteria detected in the lymphoid tissues of fish, however, indicate that the health of the fish has been compromised, even though no external indicators of poor condition may be noticeable. *Y. ruckeri* behaves in this manner and is sometimes described as establishing a 'carrier state', although in the strictest definition of this phrase this is arguable. Throughout the farm cycle, periodic stress factors may move the balance of advantage towards the pathogen, as the performance of the immune system and other physiological factors are reduced to suboptimal levels. Fish carrying *Y. ruckeri* become sluggish, reduce diet intake and may have a darkening of the skin. Factors known to promote subclinical and clinical infection by *Y. ruckeri* are, predictably, handling, grading and excessive stocking densities. When infection occurs in the absence of these factors, suspended organic matter in the water, coupled with high temperatures and consequentially low oxygen, along with exposure to low levels of chemicals in the water supply, may promote clinical disease (Bullock and Snieszko, 1975; Knittel, 1981). In areas where *Y. ruckeri* is endemic, the farm cycle is characterized by rapid changes in the external appearance of the stock and persistent low mortalities from ERM. Correction of the environmental factor(s) responsible may restore the fish to health, although an antibiotic may be needed to reduce the level of loss if condition has deteriorated too far. Acute infections, if not checked rapidly, can vary between 30 and 70% of the stock. As the fish become larger, chronic, slower infections are more characteristic, but these too may reach epizootic proportions if mishandled.

Species affected and geographic distribution

Enteric redmouth is predominantly a disease of salmonids. It has also been found to cause disease in an increasingly diverse range of fish hosts, and has been isolated from other apparently healthy animals. The major fish species infected and geographic distribution of *Y. ruckeri* are shown in Table 13.1. Following the first recognition of *Y. ruckeri* in Idaho, other workers began to identify the pathogen elsewhere. McDaniel (1971) recorded its presence in most of the Rocky Mountain area of the western USA, including Alaska, and Wobeser (1973) found it in Canada. Much of this early apparent spread was probably genuine and resulted from the dissemination of fish throughout this area in the absence of strict controls and monitoring schemes. Introduction of the disease to Europe in 1983 and its subsequent spread probably occurred in the same way, and it is now known to be present over large areas of the USA and Europe (Denmark, France, Germany, Italy, Norway and the UK). It is also present in Australia (Bullock *et al.*, 1978b; Green and Austin, 1983), South Africa (Bragg and Henton, 1986) and Chile, all of which received imported salmonid eggs and fry from infected areas. However, there is some variation in the strains and serotypes within the genus associated with geographical areas and it is likely that its natural distribution throughout temperate fresh water is more widespread than was at first thought. Considering the distribution throughout temperate waters in Europe, USA, Africa, Asia and Australia (Table 13.1), it is probably a safe assumption that *Y. ruckeri* will occur in fresh temperate waters wherever there are salmonid fish.

Diagnosis

Provisional diagnosis of *Y. ruckeri* infection in fish is often made on the basis of experience at a particular site, combined with the clinical signs in the fish. However, as indicated above, these are typical of many other Gram-negative septicaemias; even the frequently cited reddening of the mouth may be absent and, in any case, is seen in other types of infection. Additionally, small fish may die without clinical signs. Confirmation of *Y. ruckeri* infection is made most easily by culture from tissues, particularly the spleen, heart and kidney. Widely available media, such as tryptone soya agar (TSA), sometimes supplemented with 5% blood, are the most effective. The use of a pre-culture procedure, where the tissue suspected is incubated in liquid media (tryptone soya broth [TSB] or TSB + blood) at 18°C for up to 48 h, may increase the number of positives (Daly and Stevenson, 1985). If required, confirmation can be obtained by non-lethal sampling (Noga *et al.*, 1988). The regular cyclical shedding of *Y. ruckeri* that has been reported to occur from the intestinal tract of carrier fish, however, may hinder isolation (Busch and Lingg, 1975). Attempts have been made to produce semi-selective diagnostic media to aid culture (Waltman and Shotts, 1984; Rodgers, 1991b) but, as with most diagnostic selective media, growth of the target organism may be reduced in addition to the desirable reduction in growth of other species. Additionally, the requirement of this medium that Tween 80 should be hydrolysed is not met by many isolates, complicating its use (Austin and Austin, 1993). More recently, differentiation based on virulence characteristics has been used successfully (Furones *et al.*, 1993) but, in general, standard TSA is effective and preferred for isolation (Hastings and Bruno, 1985; Rodgers and Hudson, 1985). Identification based on biochemical attributes (see Table 13.2) and laboratory mini-kits, such as API 20E, is also used successfully (Santos *et al.*, 1993). However, additional tests may be required if an exact definition of the species is required, since closely related species such as *Hafnia alvei* would not be differentiated. For immunodiagnostic methods, choice may be made between monoclonal and polyclonal reagents in the protocols, although, for commercial purposes, monospecific polyclonals tend to offer higher antibody titres than MAbs purified from hybridoma supernatants. Antigen detection, using specific antibodies in enzyme-linked immunosorbent

Table 13.1. Geographic distribution of *Yersinia ruckeri* and species affected. Table is based on published records of isolation of *Y. ruckeri*.

Region	Isolated from		
Country/state	Common name	Genus, species	Reference
Europe			
Bulgaria	Rainbow trout	*Oncorhynchus mykiss*	Gelev *et al*. (1984)
Croatia	Rainbow trout	*Oncorhynchus mykiss*	Oraic *et al*. (2002)
	Rudd	*Scardinius erytnropthalmus hesperidicus*	Popovic *et al*. (2001)
Czechoslovakia	Rainbow trout	*Oncorhynchus mykiss*	Vladik and Prouza (1990)
Denmark	Rainbow trout	*Oncorhynchus mykiss*	Dalsgaard and Madsen (2000); Pedersen *et al*. (2008)
Finland	Whitefish	*Coregonus peled* *Coregonus muksun*	Rintamaki *et al*. (1986)
	Perch	*Perca fluviatilis*	Valtonen *et al*. (1992)
	Roach	*Rutilis rutilis*	
	Atlantic salmon	*Salmo salar*	
	Rainbow trout	*Oncorhynchus mykiss*	
France	Rainbow trout	*Oncorhynchus mykiss*	Lesel *et al*. (1983)
	Minnow	*Pimephales promelas*	Michel *et al*. (1986)
	Sturgeon	*Acipenser baeri*	Vuillaume *et al*. (1987)
Germany	Rainbow trout	*Oncorhynchus mykiss*	Fuhrmann *et al*. (1984)
	Brown trout	*Salmo trutta*	
	Carp	*Cyprinus carpio*	
	Eel	*Anguilla anguilla*	
Greece	Rainbow trout	*Oncorhynchus mykiss*	Savvidis (1991)
Hungary	Trout	Not specified	Csaba *et al*. (1991)
Italy	Trout	Not specified	Battisti *et al*. (2008)
Norway	Atlantic salmon	*Salmo salar*	Sparboe *et al*. (1986)
Poland	Rainbow trout	*Oncorhynchus mykiss*	Kozinska and Pekala (2004)
Portugal	Rainbow trout	*Oncorhynchus mykiss*	Sousa *et al*. (2001)
Romania	Salmonids	Not specified	Dascalescu *et al*. (2003)
Spain	Rainbow trout	*Oncorhynchus mykiss*	Romalde *et al*. (1994b); Fouz *et al*. (2006)
Switzerland	Rainbow trout	*Oncorhynchus mykiss*	Meier (1986)
Turkey	Rainbow trout	*Oncorhynchus mykiss*	Timur and Timur (1991); Saglam *et al*. (2006); Ozer *et al*. (2008)
UK	Rainbow trout	*Oncorhynchus mykiss*	Roberts (1983); Frerichs and Collins (1984); Frerichs *et al*. (1985); McCormick and McLoughlin (1993)
	Atlantic salmon	*Salmo salar*	Dear (1988)
	Otter	*Lutra lutra*	Collins *et al*. (1996)
North America			
Canada	Burbot	*Lota lota*	Dwilow *et al*. (1987)
	Cisco	*Coregonus artedii*	Stevenson and Daly (1982)
	Rainbow trout	*Oncorhynchus mykiss*	Wobeser (1973)
Alaska	Salmon	*Oncorhynchus* spp.	McDaniel (1971)
Arizona	Trout and salmon		McDaniel (1971)
Arkansas	Channel catfish	*Ictalurus punctatus*	Danley *et al*. (1999)
California	Trout and salmon		McDaniel (1971)

(*Continued*)

Table 13.1. *Continued.*

Region	Isolated from		
Country/state	Common name	Genus, species	Reference
Colorado	Trout and salmon		McDaniel (1971)
Idaho	Rainbow trout	*Oncorhynchus mykiss*	Ross *et al.* (1966)
Nevada	Trout and salmon		McDaniel (1971)
Oregon	Chinook salmon Coho salmon	*Oncorhynchus tschawytscha* *Oncorhynchus kisutch*	McDaniel (1971); Arkoosh *et al.* (2004)
South Carolina	Brown trout	*Salmo trutta*	Arias *et al.* (2007)
Utah	Trout and salmon		McDaniel (1971)
Washington	Chinook salmon Coho salmon	*Oncorhynchus tschawytscha* *Oncorhynchus kisutch*	Arkoosh *et al.* (2004)
West Virginia			Bullock *et al.* (1978a,b)
Wyoming	Trout and salmon		McDaniel (1971)
New England (Maine, Vermont, Connecticut, Mass., New Hampshire, Rhode Is.)	Atlantic salmon	*Salmo salar*	Ford *et al.* (1998); Cipriano and Coll (2005)
South America			
Peru	Rainbow trout	*Oncorhynchus mykiss*	Bravo and Kojagura (2004)
Chile	Atlantic salmon	*Salmo salar*	Troncoso *et al.* (1994)
	Carp	*Cyprinus carpio*	Enriquez and Zamora (1987)
Asia			
China	Bighead carp Silver carp	*Aristichthys nobilis* *Hypophthalmichthys molitrix*	Xu *et al.* (1991)
India	Carp	*Catla catla, Cirrhina mrigala, Labeo rohita*	Manna *et al.* (2003)
	Rainbow trout	*Oncorhynchus mykiss*	Mandeep *et al.* (1995)
(Andaman Islands)	Carp	*Cirrhina mrigala*	Shome *et al.* (1996)
Singapore	Goldfish	*Carassius auratus*	McArdle and Dooley-Martin (1985)
Oceania			
Australia	Salmonids		Bullock *et al.* (1978b); Llewellyn (1980); Green and Austin (1983)
Africa			
South Africa	Rainbow trout	*Oncorhynchus mykiss*	Bragg and Henton (1986)
Egypt	Nile tilapia	*Oreochromis niloticus*	Eissa *et al.* (2008)
Middle East			
Iran	Rainbow trout	*Oncorhynchus mykiss*	Soltani *et al.* (1999); Akhlaghi and Sharifi (2008)

assay (ELISA) formats, has been used both in research and commercially in plate and rapid 'on-stick' formats. Antibody detection using latex-agglutination has been used to determine subclinical infections (Romalde *et al.*, 1995).

A specific diagnostic PCR for *Y. ruckeri* was developed based on a unique band identified during randomly amplified polymorphic DNA analysis. The PCR based on this fragment was specific for *Y. ruckeri* in mixed populations, yet was common to all

Table 13.2. Biochemical attributes of *Y. ruckeri*.

Test	Reaction	Test	Reaction
Substrate utilization		*Enzymatic reaction*	
Adonitol	–	Aesculinase	–
Arabinose	–	Arginine dihydrolase	+ (59%); + d (41%)
Cellobiose	–	Beta-galactosidase	+
Citrate (Simmons)	+ (3%); + d (94%); – (3%)	Caseinase	+ (51%)
Dulcitol	–	Catalase	+
Erythritol	–	Chitinase	–
Esculin	–	Chondroitin sulfatase	–
Galactose	–	Cytochrome oxidase	–
Glucose	+ (17% + gas)	Deoxyribonuclease	–
Glycerol	+ (70%); + d (15%)	Elastinase	–
Inositol	–	Fibrinolase	–
Lactose	–	Gelatinase	+ (52%); + d (41%); – (6%)
Maltose	+	Hyaluronidase	–
Mannitol	+	Lecithinase	+ (81%)
Mannose	+ (97%)	Lipase (Tween 20)	+
Melibiose	–	Lysine decarboxylase	+ (88%)
Methyl red	+ (79%)	Ornithine decarboxylase	+
Mucate	–	Pectinase	–
Raffinose	–	Phenylalanine deaminase	–
Rhamnose	–	Phosphatase	–
Salicin	–	Ribonuclease	–
Sorbitol	+ (25%); + d (75%)	Tributyrinase	–
Starch	–	Urease	–
Sucrose	–	*Other reactions*	
Trehalose	+	Growth in KCN	+ (24%)
Triple sugar iron	Alkaline slant/acid butt	Haemolysis	alpha
Xylose	–	H_2S production	–
		Indole production	–
		Motility	+ (82%)
		Nitrate reduction	+ (85%); + d (12%); – (3%)
		Nitrite reduction	–

d, differential (sometimes positive, sometimes negative).

serotypes tested. The PCR was capable of detecting the pathogen in tissue samples and would therefore appear to be quite useful (Argenton *et al.*, 1996). A different PCR reaction has been employed successfully to identify *Y. ruckeri* in experimentally and naturally infected fish (Gibello *et al.*, 1999) and PCR has been used to detect *Y. ruckeri* by non-lethal sampling of blood (Altinok *et al.*, 2001). Subsequently, other PCR methods have been developed based on amplification of various genes and with some refinements to increase sensitivity or to allow multiplexing (Temprano *et al.*, 2001; Taylor and Winton, 2002; Wilson *et al.*, 2002; Saleh *et al.*, 2008).

The current author would recommend isolation in pure culture, followed by identification by amplification and sequencing of the 16S rRNA gene using the universal bacterial primers 27F and 1492R (Lane, 1991) as the gold standard, providing a very robust diagnostic *where pure cultures can be achieved*. Sequencing is relatively inexpensive

and very fast, returning results overnight in most cases. This, in combination with case history, clinical signs and a diagnostic PCR, should provide sufficient corroborative evidence and avoids the need for extensive biochemical testing of the isolate.

Control and treatment

Transmission of the disease

Early work and descriptions of the disease were made by those working in salmonid aquaculture, leading to the concept of ERM as primarily a disease of salmonids. Confirmed clinical outbreaks were seen in both trout (rainbow and steelhead [*Oncorhynchus mykiss*], cutthroat, brown [*Salmo trutta*] and brook [*Salvelinus fontinalis*]) and in salmon (coho [*O. kisutch*], sockeye [*O. nerka*] and Atlantic [*S. salar*]) (Busch, 1982). Early epizootics in these species have been described in detail by Ross *et al.* (1966), Rucker (1966) and Busch (1973). More recently, it has been recognized that the host range of *Y. ruckeri* is more diverse, and a list of the species from which it has now been isolated is given in Table 13.1. Whether it is capable of a saprophytic existence as a part of the environmental microbiota outside a host is a matter of debate; Rucker (1966) suggested that this may be the case, but later authors, such as Klontz and Huddleston (1976), argued against this. Their findings reflected those seen for many other Gram-negative coliforms, including *Escherichia coli* and *A. salmonicida*, where the survival in clean water in a laboratory experiment may be a matter of hours, but may be prolonged for 2–3 months in sediments or where there is organic matter present (Romalde *et al.*, 1994a). This allows transmission from fish to fish under natural conditions without the need to propose that the organism has lived saprophytically outside an animal host. However, as discussed above, the ability to survive in biofilms associated with algae and various solid supports associated with fish farms indicates that *Y. ruckeri* may persist in the environment, providing a reservoir of infection outside the fish (Coquet *et al.*, 2002a,b). In spite of this, the prime source of infection is generally considered to be the shedding of large numbers of bacteria from carrier or infected fish in the faeces. Such fish do not normally shed quantities capable of causing infection, unless stressed (Hunter *et al.*, 1980). The level of carrier fish in a population depends on the criteria and methods used. Busch and Lingg (1975) demonstrated that, 45 days post-infection, 25% of rainbow trout carried *Y. ruckeri* in the lower intestine, and with no clinical signs. The preculture/culture method used in this case is sensitive and the data may reflect a situation in fish following the first serious epizootic in a population. However, where a detailed investigation is made using culture, immunological and molecular methods together in a population grown under normal farm conditions for several months, a much higher proportion of individuals are found to be positive and it is not improbable that all are actually carrying the pathogen. *Y. ruckeri* has also been isolated from broodstock, which is frequently infected, but transmission through eggs where disinfection has been carried out properly has not been reported (Dulin *et al.*, 1976). Conditions predisposing populations to clinical infection relate primarily to stress. Healthy laboratory populations can withstand exposure to high numbers of cells without succumbing to clinical disease (Ross *et al.*, 1966). Infection may occur where fish are obese through poor feeding regimes, but poor water quality is the prime cause. Common causes are high ammonia and low oxygen due to poor water flow or excessive stocking densities, high temperatures (which also reduce the oxygen-carrying capacity of the system), or the presence of a high level of suspended organic and siliceous matter (Bullock and Snieszko, 1975). When these conditions are marginal, handling stress may trigger infections where the fish would have remained healthy if left untouched. The expectation of trouble, therefore, is in summer conditions where temperatures rise and water flows are reduced. The peak is considered to be 15–18°C and monitoring oxygen and temperatures daily is an effective warning system where *Y. ruckeri* is endemic. Infections have not been reported below 10°C.

Prevention and control

Prevention of ERM outbreaks is achieved by avoiding the introduction of infected stock and ensuring that all imported eggs have been disinfected properly. In areas where *Y. ruckeri* is endemic, prevention is achieved by avoiding those husbandry conditions described above which promote infection, although in many areas where adequate summer water flow cannot be guaranteed and high temperatures occur, some degree of infection may be inevitable. The problem can be contained by the reduction of handling, the lowering of densities, treatment of water (Liltved *et al.*, 1995; Liltved and Landfald, 1996) and the use of vaccination (Bruno and Munro, 1989; Rodgers, 1991a,b). Each of these factors lowers the threshold at which a clinical outbreak may occur. If a serious outbreak does occur, antibiotics may need to be administered.

ERM was one of the first diseases for which an effective commercial fish vaccine was developed (Busch, 1978; Cossarini-Dunier, 1986a) and vaccination was widely employed as an effective aid in its control. Fish vaccination has been reviewed (Sommerset *et al.*, 2005) and *Y. ruckeri* vaccines reviewed specifically by Stevenson (1997). Most commercial vaccines are bacterins prepared from virulent isolates, some using whole bacterial cells and others exposing internal bacterial cell components by lysis at pH 9.8. Most commercial vaccines use the whole-cell serotype, serovar I (Hagerman strain), which is the cause of the majority of disease outbreaks. Some outbreaks have been attributed to serovar II (Cipriano *et al.*, 1986), but vaccines formulated against only serovar I appear to be cross-protective in North American aquaculture, preventing infection by either type. The situation in Norway and South America, where other variants have been reported, may be different. In Norway, although commercial bacterins based on serovar I (Hagerman) gave adequate protection against Norwegian serogroup III, protection was significantly lower against Norwegian serogroups I and II. Addition of Norwegian serogroup II bacterin to the commercial preparation enhanced protection against serovar I and Norwegian serogroup II (Erdal, 1989). Commercial vaccines are administered by intraperitoneal (intracoelomic) injection or immersion. In the latter case, the major source of uptake is the gill, where antigen-trapping cells have been detected (Torroba *et al.*, 1993) and high levels of antibody-secreting cells are detected after immersion vaccination (dos Santos *et al.*, 2001).

Under field conditions, the effectiveness of ERM vaccines may vary. In situations where fish are highly stressed through inappropriate handling, poor water quality, overstocking or poor nutrition, vaccines rarely prevent disease completely as the immune system is also affected by these conditions (Pickering and Pottinger, 1987). Even so, a marked difference is seen between control and unvaccinated stocks (Tebbit *et al.*, 1981). Where fish are grown under less severe conditions, the incidence of disease in vaccinated populations is frequently at a level sufficiently low for no medication to be required throughout the growth cycle, and vaccination is therefore very cost-effective (Horne and Robertson, 1987). Lately, novel variants of *Y. ruckeri* have been isolated, including non-motile variants of serotype O1, from vaccinated fish (Austin *et al.*, 2003; Fouz *et al.*, 2006). The observation that this has now occurred in separate countries (the UK and Spain), and that both strains come from vaccinated stock and are non-motile, is highly supportive of the importance of flagellar antigens in current vaccines (Stevenson, 1997). It appears that protective immunity in the host is driving evolution of novel variants of the pathogen that are not recognized by immunized animals. It is surprising that it has taken 30 years of widespread vaccination before this phenomenon has occurred and is testament to the efficacy of the killed bacterin vaccines for this disease. Similar phenomena have occurred very quickly against other fish pathogens, with far less widespread use of vaccination (Bachrach *et al.*, 2001; Nawawi *et al.*, 2008, 2009). Improved knowledge of key virulence factors (Fernandez *et al.*, 2002, 2003, 2004,

2007a,b; Mendez *et al.*, 2009) and the importance of cellular immunity in protective response will be key in formulating the next generation of vaccines (Wiens *et al.*, 2006; Raida and Buchmann, 2008a,b, 2009).

Antimicrobial therapy

Y. ruckeri is generally sensitive to the range of antibiotics used routinely in aquaculture (Inglis and Richards, 1991; Schmidt *et al.*, 2000; Michel *et al.*, 2003). Two or three treatments are commonly needed throughout a summer season and during this time resistance may easily develop (Tebbit *et al.*, 1981; Ceschia *et al.*, 1987; Rodgers, 2001). The compounds used most regularly for control of *Y. ruckeri* infections are sulfamerazine, oxytetracycline, tribrissen and oxolinic acid (Inglis *et al.*, 1995), although the latter has now been deregistered and replaced with sarafloxacin. Amoxicillin is also licensed in the UK. More recent introduction of florfenicol commercially has provided another tool in the arsenal of antimicrobials available and, in a survey of French isolates, all isolates would be considered susceptible with MICs of 8 μg/ml or less (Michel *et al.*, 2003). Resistance to antimicrobials does occur in *Y. ruckeri* with extended or repeated use (Tebbit *et al.*, 1981), and resistance to oxytetracycline, oxolinic acid and sulfadiazine/trimethoprim (5:1) has been induced by repeated passage *in vitro* (Rodgers, 2001). Plasmid-mediated multiple resistance to oxytetracycline and sulfonamides has been reported (De Grandis and Stevenson, 1985). *gyrA* mutations have been detected in strains with reduced susceptibility to quinolones (Gibello *et al.*, 2004) and a class C beta lactamase has been characterized that would render amoxicillin ineffective for treatment of ERM caused by strains carrying the gene (Mammeri *et al.*, 2006). Increasing resistance to antimicrobials with increasing use is almost a certainty, and with the high prevalence of mobilizable resistance elements throughout the genus *Yersinia*, including *Y. ruckeri* (De Grandis and Stevenson, 1985; Welch *et al.*, 2007), the risks to fish farm and human health are high. This, coupled with the general move towards antibiotic removal from food animal production, should be driving towards alternative control methods but is dependent on the choices made by veterinarians and farmers and the availability of effective preventative products such as vaccines at a price that farmers can afford.

Probiotics

The proposed use of potential beneficial bacteria or probiotics to manage human and animal health dates back to the early 1900s (Morelli, 2000). Probiotic use in fish is patchy, but is gaining some momentum. Hypothetically, probiotics may colonize the gastrointestinal tract of target organisms, occupying niches which may prevent establishment of pathogens, stimulating non-specific immunity, or inhibiting pathogens directly by production of antimicrobial compounds. Additionally, probiotic bacteria may improve water quality. Against this background, there are numerous characteristics of *Y. ruckeri* infection that make use of probiotic bacteria seem feasible. *Y. ruckeri* infections are exacerbated by poor water quality; thus, improvement of the microbiological quality of the water may be beneficial. During infection, high levels of *Y. ruckeri* are found in the gastrointestinal tract and carrier fish contain high numbers of *Y. ruckeri* in the faeces, which is thought to be a major route of transmission of the disease through the population. Recent research supports the benefit of probiotic use for control of *Y. ruckeri* infection (Raida *et al.*, 2003; Brunt *et al.*, 2007).

Yersinia ruckeri

Y. ruckeri is a member of the family *Enterobacteriaceae* within the *gammaproteobacteria* subdivision. Generally of coccoid-rod cell morphology, *Y. ruckeri* cells are slightly curved, 1.0 μm in diameter and 2–3 μm in length, though culture for 48 h or longer results in long filamentous cells.

Taxonomy

Historically, placement of the organism in the genus *Yersinia* has been controversial (Austin and Austin, 1993). Ross *et al.* (1966) initially placed the bacterium in the *Enterobacteriaceae* based on morphological and biochemical attributes, but within this group its allocation to either *Yersinia* or *Serratia* was uncertain. Serological and biochemical studies had also indicated relationships with *Arizona* (= *Salmonella*) *arizonae* (Ewing *et al.*, 1978) and serological cross-reactions with *H. alvei* were also reported (Stevenson and Daly, 1982). Deoxyribonucleic acid (DNA) homology indicated equal relationships to *Y. enterocolitica* (30–31%), *Y. pseudotuberculosis* (30–31%), *S. marcescens* (24–28%) and *S. liquefaciens* (31%). Whereas the latter two species have been isolated from fish farm situations and causing disease, neither *Yersinia* species has been associated with fish health problems. Green and Austin (1983) showed that there was a greater phenotypic relationship between *Y. ruckeri* and *S. arizonae* than with either *Y. enterocolitica* or *Y. pseudotuberculosis*, while others suggested that *Y. ruckeri* might be considered more properly a new species within the *Enterobacteriaceae* (Bercovier and Mollaret, 1984). Its elevation to species status and designation as *Y. ruckeri* was made in 1978 (Ewing *et al.*, 1978), and its acceptance was strengthened by analysis of the DNA base composition (mol. % G + C) (Ewing *et al.*, 1978). *Y. ruckeri*, at 47.5–48%, is clearly different from *Serratia* (52–60%) and *S. arizonae* (50–53%), whereas it is close to other *Yersinia* species (46–50%) (De Grandis *et al.*, 1988). Nevertheless, its placement within *Yersinia* based on DNA hybridization, biochemical attributes and 'lifestyle' still appears anomalous to some researchers and a new genus has been proposed (Bercovier and Mollaret, 1984), except that *Y. ruckeri* exists as a single, relatively homogeneous set of isolates.

The use of molecular techniques now supports the placement of *Y. ruckeri* within the genus *Yersinia* based on 16S rRNA gene homology (Ibrahim *et al.*, 1993). With the discovery and characterization of more new species in the genus, the phylogenetic position of *Y. ruckeri* will likely become more robust. Recent analysis of the 16S rRNA gene of *Y. ruckeri* based on sequences available in the NCBI database places *Y. ruckeri* within *Yersinia*, with close affiliation to *Y. kristensenii* with high bootstrap support (Fig. 13.1).

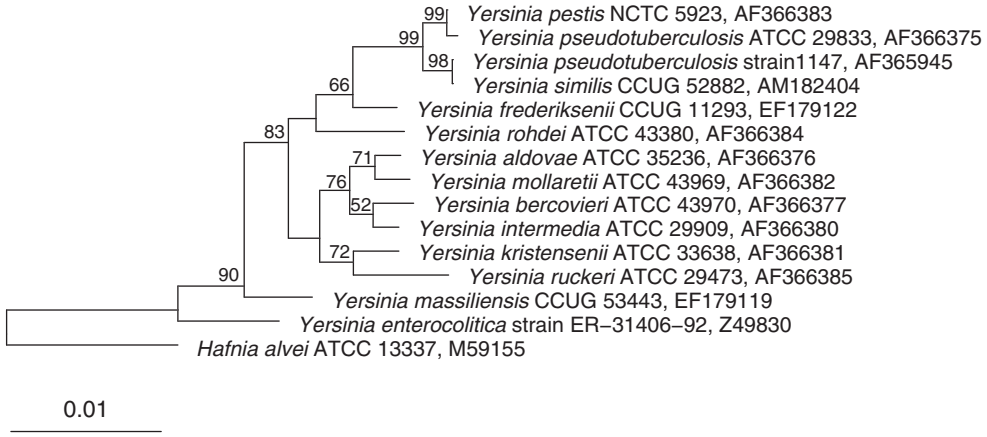


Fig. 13.1. Evolutionary distance dendrogram of the genus *Yersinia* based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method with the Jukes–Cantor algorithm using 1461 nt of data in the ARB software package (Ludwig *et al.,* 2004). Bootstrapping was sampled 1000 times. Branch points supported by ≥ 50% of bootstraps are indicated. The bar represents 1% estimated sequence divergence. *Hafnia alvei* is the outgroup.

In contrast, phylogenetic analysis based on the nucleotide sequences of four housekeeping genes placed *Y. ruckeri* as a deep-branching member of the genus (Kotetishvili *et al.*, 2005). As the rates of change for the 16S rRNA gene are slow (Woese, 1987), this gene can be used as a molecular chronometer/clock, making molecular phylogeny based on 16S sequences extremely reliable. In contrast, housekeeping genes may be transferred laterally from one member of the *Enterobacteriaceae* to another, or are susceptible to changes from selective pressures and thus lead to skewing. Based on this potential genetic flexibility, multi-locus sequence typing based on housekeeping genes is more useful for determining intraspecific variability rather than the taxonomic placement of a species within a particular genus. Thus, the present phylogenetic position based on 16S rRNA gene homology (Fig. 13.1) is reliable and is likely to become more so as a greater number of 16S sequences for the *Yersinia* genus become available.

Serology and intraspecific variation

There is a high degree of intraspecific variability based on sugar utilization (biotypes), outer membrane protein (OMP) profiles, lipolysaccharide O-antigen and flagellar antigens, and these may or may not be associated with differing cross-reactivity of whole cells with antisera.

The basic biochemical attributes of *Y. ruckeri* have been described frequently (Ross *et al.*, 1966; Wobeser, 1973; O'Leary, 1977; Bullock *et al.*, 1978b; Ewing *et al.*, 1978; Stevenson and Daly, 1982; Bercovier and Mollaret, 1984; Hastings and Bruno, 1985; Davies and Frerichs, 1989; Austin and Austin, 1993) and the most common of these reactions are shown in Table 13.2. Approximately 80% of isolates are motile, using seven or eight peritrichously arranged flagella, but at 9°C or lower the flagella do not function and at 35°C flagella are absent and the cells are not motile (O'Leary, 1977). Cells cultured longer than 48 h are poorly motile. *Y. ruckeri* ferments glucose, maltose, mannitol, mannose and trehalose, and it also uses methyl red and glycerol (Table 13.2). However, one of the most reported attributes is the varying ability of differing strains to ferment sorbitol and this has been proposed for biotyping. O'Leary (1977) reported that this trait was characteristic of serovar II and could be used to distinguish it from serovar I. However, subsequent studies demonstrated that some sorbitol fermenting isolates cross-reacted serologically with serovar I. Indeed, 32% of strains tested fermented sorbitol (De Grandis *et al.*, 1988), and Austin and Austin (1993) suggested that this trait might be plasmid-mediated.

Although the biochemical attributes of isolates from different areas are mostly uniform, serological variations exist based on lipopolysaccharide O-antigens (Davies, 1990; Romalde *et al.*, 1993; Bateman *et al.*, 1996), OMPs (Davies, 1991c,d) or flagellar antigens recognized on the cell surface by antisera, usually raised in rabbits. Serological variation is complex and numerous methods have been applied, resulting in conflicting nomenclature. Originally, five major serovars were recognized, based on whole-cell reactions and described as type I (Hagerman), the most virulent; type II (O'Leary), avirulent; type III (Australian), avirulent; serovar IV, avirulent; and serovar V, avirulent (Stevenson and Airdrie, 1984; Daly *et al.*, 1986). Later work showed that many infections were associated with type II, which, in some instances, is as virulent as type I. Further complication arose following the identification of the Australian salmon blood spot bacterium (Llewellyn, 1980) as *Y. ruckeri* (De Grandis *et al.*, 1988), which has now been included in serovar III, although there is cross-reaction with serovar I antisera. Current nomenclature, based on whole-cell serology, is summarized in Table 13.3 (adapted from Stevenson *et al.*, 1993). Six whole-cell serovars (I–VI) have been proposed (Stevenson *et al.*, 1993), although DNA homology analysis has discounted serovar IV (De Grandis *et al.*, 1988). However, these major whole-cell serotypes may be further subdivided, according to the methodology applied. Lipopolysaccharide

Table 13.3. Whole-cell serotypes of *Y. ruckeri* (after Stevenson *et al.*, 1993).

Serovar	Designation	Notes
I	Hagerman	Cross-reacts with III and V
II	Oregon	Includes at least two further groups determined with absorbed sera
III	Australian	Includes I' (Salmonid blood spot)
IV		Excluded by DND:DNA homology
V	Colorado	
VI	Ontario	

Table 13.4. Lipopolysaccharide O-antigen serotypes of *Y. ruckeri* (after Stevenson *et al.*, 1993).

O-antigen serogroup	Whole-cell serovar
O:1	I, III
O:2	IIa
O:3	IIb
O:4	IIc
O:5	V
O:6	VI

Table 13.5. Serotyping scheme proposed by Romalde *et al.* (1993).

Serotype	Subgroup	Former serovar
O1	a	I
	b	III
O2	a	II
	b	II
	c	II
O3	n/a	V
O4	n/a	VI

n/a, not applicable.

profiles obtained using electrophoresis and immunoblot have provided a further basis for classification, and six O-serogroups, based on LPS O-antigen, have been identified (Table 13.4; Stevenson *et al.*, 1993). Cross-reaction between whole-cell serovars I and II appears to result from similar LPS, although serological differences are evident when absorbed sera are used (Stevenson *et al.*, 1993). An additional scheme for serological classification has been proposed by Romalde *et al.* (1993), based on studies using absorbed sera and antigenic determinants (LPS and membrane proteins). In this study, four serogroups were suggested (Table 13.5), incorporating the six predominant serovars proposed by Stevenson *et al.* (1993). Regardless of serotype, DNA restriction fingerprinting showed that *Y. ruckeri* was highly homogeneous (Romalde *et al.*, 1993). However, plasmid profiling showed that only isolates of serogroup O1 contained a 50 MDa plasmid (Romalde *et al.*, 1993). Further complication of serological nomenclature arises with the Norwegian classification of *Y. ruckeri* into serogroups I, II and III. Many Norwegian isolates appear to be related to serovar I, based on LPS profiles, but differ serologically (Ibrahim *et al.*, 1993). Furthermore, cross-protection studies suggest antigenic similarities between Norwegian serogroup III and Hagerman serovar I, while Norwegian serogroups II and III are different (Erdal, 1989).

Further elucidation of the molecular basis for whole-cell serological reactions has been attempted by separating out cell-wall components other than LPS. OMPs can be separated from other Gram-negative cell-wall components by differential solubilization in detergents. OMP profiling of *Y. ruckeri* has been used with some success, based on variation in mobility and number (2 or 3) of the major OMPs lying between 36.5 kDa and 40.5 kDa, after extraction with Sarkosyl (Davies, 1991c) and OMPs clearly account for some degree of the serological variation detected in whole cells with rabbit antisera. Analysis of these major OMPs indicated

that 5 OMP types could be identified in SDS-PAGE gels (Davies, 1991c). Using this scheme, of the 135 isolates of *Y. ruckeri* from Canada, the USA, Australia, South Africa, mainland Europe and the UK, most European isolates were of OMP type 3, while isolates from the UK fell into OMP type 1, similar to the Hagerman isolate. US isolates were distributed among OMP types 1–3, while representatives of all 5 OMP types were isolated in Canada (Davies, 1991c).

Attempts to clarify intraspecific variability by defining clonal groups for *Y. ruckeri*, similar to those proposed for *E. coli*, were proposed based on biotyping and OMP profiling (Davies, 1991b). Six clonal groups were proposed, based on five OMP types and two biotypes, using isolates of *Y. ruckeri* from diverse geographical areas and falling into 0-serotypes 01, 02, 05, 06 and 07 (Davies, 1991d). Using this complex scheme, Davies found that clonal groups 1, 2 and 4 were unique to Australia, the UK and Norway, respectively. While Australian isolates were also found in the clone 3 group, none were found in the clone 5 'Hagerman' group, confirming earlier contention that the virulent 'Hagerman' isolate was exotic to Australia (Humphrey *et al.*, 1987; Davies, 1991d).

Intraspecific variability remains a complex issue. Serology provides the most relevant information in terms of what the host animal 'regards' as different and has greatest significance in terms of vaccine development. However, there are numerous problems associated with the methodology, including variability between laboratories, variability between batches of antisera and the need to incorporate increasing numbers of reference strains and antisera to 'benchmark' or calibrate the serological reactions. For epidemiological monitoring, the connection to the hosts' immune perception of the organism is less important provided the methods used are sufficient to distinguish between strains and not so discriminatory that irrelevant variability is found among simultaneous isolations from the same sites and hosts. A few molecular techniques lend themselves to this type of work, yet few have been attempted with *Y. ruckeri*. Restriction digestion of genomic DNA with EcoR1 and BamH1 followed by analysis separation on acrylamide gels did not differentiate between strains that could be distinguished on the basis of LPS pattern and O-antigen serology (Romalde *et al.*, 1993). This probably reflects on the choice of restriction enzymes and analysis techniques; a single restriction enzyme digestion followed by pulsed-field separation of the digested fragments is more likely to produce usable information. Indeed, PFGE has been used to analyse serovar O1 isolates from Italy; while discrimination between three isolates from Italy was not possible, there were differences in the profiles of the three isolates compared with ATCC29743 (serovar O1) and compared with a serovar O2 strain (Lucangeli *et al.*, 2000). Further work on PFGE profiling is required using differing restriction enzymes and, more importantly, more strains with good provenance of origin. This is a powerful technique that has been used with epidemiological relevance to type numerous fish pathogens, including *Vibrio anguillarum* (Skov *et al.*, 1995) and *Streptococcus iniae* (Facklam *et al.*, 2005; Nawawi *et al.*, 2008). Such techniques will be essential as new serotypes and biogroups emerge under selective pressure from vaccination (Austin *et al.*, 2003).

Virulence factors

The early development, adoption and success of vaccination for prevention and control of *Y. ruckeri* infection perhaps reflects the dearth of research into virulence mechanisms until very recently, when further research was mitigated by the emergence of novel biotypes that were not controlled by current killed bacterins. Although sparse, there is some early research on virulence that provides a useful basis over which to explore more recent, comprehensive molecular studies that have been reviewed (Fernandez *et al.*, 2007a).

Survival outside the host

It has been postulated that *Y. ruckeri* is an obligate pathogen, transmitting only between

wild or carrier fish or other putative live vectors (Austin and Austin, 1993). However, several studies indicate that *Y. ruckeri* is capable of surviving in both water and sediments for more than 3 months, with survival higher in sediments than in water (Romalde *et al.*, 1994b). Survival in water may be dependent on salinity, as *Y. ruckeri* survives in unsupplemented fresh or brackish water (salinity 0–20 ppt) for at least 4 months, while survival is greatly reduced in water of salinity 35 ppt. Starvation survival may be facilitated by replicating the genome at the onset of starvation, with starved cells being able to carry up to six copies of the genome (Thorsen *et al.*, 1992). *Y. ruckeri* may survive outside the host in a viable but non-culturable state, with accompanying cell wall/LPS changes associated with L-forms, as in other enteric bacteria (McIntosh and Austin, 1990; Romalde *et al.*, 1994a). However no changes were detected in OMPs of *Y. ruckeri* in viable but non-culturable state in aquatic microcosms (Romalde *et al.*, 1994a). L-forms of *Y. ruckeri* could be induced in the laboratory successfully (McIntosh and Austin, 1990) and viable colonies of *Y. ruckeri* recovered from the non-culturable state in water samples by addition of culture medium (Romalde *et al.*, 1994a).

In a French trout farm, *Y. ruckeri* was found more frequently in association with algae and sediment than with water (Coquet *et al.*, 2002b), corroborating the survival observations of Romalde *et al.* (1994a). The association with sediments and microalgae was attributed to the biofilm-forming capacity of the organism, which was associated with motility. *Y. ruckeri* is also capable of forming biofilms on solid surfaces in fish farms, including PVC, fibreglass, concrete and wood (Coquet *et al.*, 2002a); however, this capability was strain-, growth phase- and surface–substrate-dependent, with concrete and wood providing the richest communities after 9 h (Coquet *et al.*, 2002a). Interestingly, immobilization on surfaces was associated with changes in OMP profiles compared with planktonic cells in either exponential or stationary phase (Coquet *et al.*, 2005). Formation of biofilms is often assisted by pili or flagella that enable the organisms to attach to the surface and move across it until they form microcolonies with other bacteria. Biofilm formation by *Y. ruckeri* correlates closely with flagellar motility, which supports this contention (Coquet *et al.*, 2002b). Thus, *Y. ruckeri* may remain viable in aquatic environments, most likely associated with sediments, algae or fish farm structures that provide anchorage for biofilm formation, providing a suitable source for re-infection of stock and repeated outbreaks of disease.

Competition for substrate and host colonization with other aquatic microorganisms should also be considered as part of a survival strategy outside the host. *Y. ruckeri* produces water-soluble antimicrobial activity capable of inhibiting *V. anguillarum*, *A. hydrophila* and *A. salmonicida* (Michel and Faivre, 1987). This is not without precedent, as strains of *Y. pestis* produce a potent *N*-acetylmuramic acid-hydrolysing bacteriocin, pesticin (Hu and Brubaker, 1974; Ferber and Brubaker, 1979; Vollmer *et al.*, 1997). However, the activity produced by *Y. ruckeri* does not inhibit other strains of *Y. ruckeri* and, as such, does not fulfil the criteria of a bacteriocin (Michel and Faivre, 1987), although it is unclear which serotypes were used in this study. Whether or not the production of antimicrobial activity confers a selective advantage on *Y. ruckeri* remains contentious, as mixed infections by *Y. ruckeri* with both *A. salmonicida* and *V. anguillarum* could be obtained experimentally in rainbow trout (Michel and Faivre, 1987), but competitive inhibition in the aquatic environment prior to host entry or in the very early stages of infection may be important.

Adhesion and entry into the host

Adhesion and entry into the host are the next steps in the establishment of an infectious disease. As with survival in the farm environment, biofilm formation and flagella are essential for this process. When *flaA*, one of the key genes required for expression of flagellae, was knocked out in *Y. ruckeri*, fish challenged with both the knockout and wild-type strains by direct intraperitoneal

injection suffered similar high levels of mortality. However, when uninfected sentinel fish were placed in the tanks with the infected fish, only those infected with the wild-type strain were able to transmit the disease to the uninfected animals; thus, flagella appear to be critical in the early stages of attachment and entry (Kim, 2000). If this process is bypassed, for example by injection, the disease progresses similarly in the absence of flagella (Kim, 2000). Clearly, further work is needed to determine the role of flagella in the entry process, but the association with attachment and biofilm formation cannot be ignored.

Attachment to host cells has been investigated using a number of different fish and mammalian cell lines and was both strain- and cell line-dependent. Using the type strain of *Y. ruckeri*, low invasion rates were detected in rainbow trout kidney (RTPK) and rainbow trout gonad (RTG) cells in culture (Kawula *et al.*, 1996). Higher invasiveness was recorded in fathead minnow epithelial cells (FHM), while *Y. ruckeri* was not capable of invading human epithelial cells (Hep-2). A positive control comprising invasive *Y. enterocolitica* was capable of invading both Hep-2 and FHM cells (Kawula *et al.*, 1996). In a study on cell surface hydrophobicity and adherence to carp epithelial cells (EPC), none of the *Y. ruckeri* strains tested (4 serotype 01, 1 from serotype 02) were capable of binding to the cells (Santos *et al.*, 1990). Adherence and invasion seem to be strain specific, and this is not correlated directly to serotype. In a study investigating adherence to CHSE214 and RTG2 cell lines, employing 12 isolates of differing host and/or geographic origin, most of the isolates adhered to CHSE214 cells to some degree; however, few strains adhered to RTG-2, with only two strains classed as strongly adherent. Both adherent strains were isolated from *O. mykiss*, one in Spain and one in the USA, with the former belonging to serotype O1a, while the latter was serotype O4 (Romalde and Toranzo, 1993). The serotype O4 isolate was also highly invasive in both cell lines. To date, no studies have elucidated pathogen-associated factors involved directly in adherence and invasion, although the requirement of flagella for biofilm formation is suggestive of their role in attachment.

Acquisition of iron

Acquisition of iron within the host is a requirement for most pathogenic bacteria where they must compete with the hosts' own high affinity iron-withholding systems such as transferrin, lactoferrin and haem compounds (Bullen, 1987; Griffiths, 1987). Four iron-regulated outer membrane proteins (IROMPs) involved putatively in iron transport were expressed under iron-limited culture conditions in a diverse array of *Y. ruckeri* strains representing all the major serotypes. No associated siderophores were detected on CAS agar, or by using biochemical assays of iron-limited spent culture supernatants for catecholate or phenolate siderophores (Davies, 1991a). Subsequently, phenolate siderophores were found in *Y. ruckeri* using CAS agar coupled with the assay for phenolate siderophores, although they were unable to cross-feed aerobactin or enterobactin-deficient indicator strains (Romalde *et al.*, 1991). More recently, an elegant molecular survey of *in vivo*-induced genes in *Y. ruckeri* detected and characterized a cluster of genes encoding a high affinity catecholate siderophore-based iron uptake system, named ruckerbactin (Fernandez *et al.*, 2004). While the gene cluster for the system showed similar organization to the enterobactin system of *E. coli*, the receptor genes had higher homology with chrysobactin receptors from *Erwinia chrysanthemi* (Fernandez *et al.*, 2004), perhaps explaining why the siderophores isolated in the previous study were unable to cross-feed enterobactin-defective mutants (Romalde *et al.*, 1991). The genes for the ruckerbactin system are controlled by both iron limitation (positive) and temperature (negative, higher at 18°C than 22°C). While both *rucC*, involved in siderophore biosynthesis, and *rupDGC*, encoding the membrane transport components, are regulated positively in response to depleted iron, *rucC* expression is induced much more strongly than the membrane transport system. Deletion mutants deficient in *rucC* did not survive well in

iron-depleted medium and had a much higher LD_{50} in infection challenge models, indicating a requirement for the siderophore in iron uptake and for full virulence in trout (Fernandez *et al.*, 2004).

Secreted toxins and other products

Crude extracellular products (ECPs) from a range of strains of *Y. ruckeri* representing the major serogroups contain caseinase, gelatinase, phospholipase, lipase and amylase activities, and are also haemolytic for fish and mammalian blood. However, only one strain from serogroup O1a produced ECPs that were cytotoxic to both fish and mammalian cells (Romalde and Toranzo, 1993). With the advent of molecular techniques, candidates for some of these pathological activities have been identified. A 47 kDa protease was purified and characterized and shown to be active in only 8 out of 14 strains assayed. Enzyme activity was divalent cation-dependent and expression was under catabolite-repression control (Secades and Guijarro, 1999). Subsequent molecular analysis of the *yrp1* gene encoding the protease determined that the enzyme was a member of the serralysin family secreted by an ABC transport system (Fernandez *et al.*, 2002, 2007a). In spite of enzyme activity only having been detected in some (azo+) strains, the *yrpI* operon was found in all strains studied (Fernandez *et al.*, 2003). The YrpI protease appeared to be involved in virulence, as deletion mutants were partially attenuated (Fernandez *et al.*, 2002). The purified protein was hydrolytic for a range of substrates, including laminin, myosin, actin, fibrinogen and gelatin (Fernandez *et al.*, 2003), perhaps accounting for the gelatinase activity recorded in crude ECPs by Romalde and Toranzo (1993).

Haemolytic activity against both fish and mammalian cells in crude ECPs (Romalde and Toranzo, 1993) may be attributed to an inducible haemolysin YhlA. Expression and export of YhlA is determined by genes *yhlB* and *yhlA* that form a single transcriptional unit and are regulated by iron limitation and temperature similarly to *rucC* (Fernandez *et al.*, 2007b), perhaps suggesting a common regulatory apparatus for these genes. Indeed, regulation of genes in response to iron depletion in most other *Yersinia* species is under Fur control (Staggs and Perry, 1992), although this regulon has not yet been identified in *Y. ruckeri*. *yhlA* and *yhlB* were detected in strains of *Y. ruckeri* isolated from fish farms in Denmark, the UK, Spain and the USA, suggesting broad, possibly universal, distribution of these genes in *Y. ruckeri* (Fernandez *et al.*, 2007b).

A further secreted product, which could fit equally well into the next section on evasion of host defences, is a putative peptidoglycan deacetylase. While there are no published data on this enzyme in *Y. ruckeri* to date, the complete cDNA sequence was deposited in the Genbank database at NCBI in 2007 by Dahiya and Stevenson of the University of Guelph (Accession EU169586). This gene and its product are particularly interesting, as the gene product is a key virulence factor vital to lysozyme resistance in streptococcal pathogens (Vollmer and Tomasz, 2000, 2002; Blair *et al.*, 2005; Davis *et al.*, 2008).

Evasion of host innate and adaptive immune defences

Resistance to (or growth in) rainbow trout normal serum is associated with virulent isolates of *Y. ruckeri* from serotype O1. In contrast, avirulent isolates of the O1 serotype are killed by normal serum, as are isolates of the O2, O5, O6 and O7 serotypes (Davies, 1991b). The host innate immune factors in normal serum include complement (activated via the alternative or lectin pathways), lysozyme, a range of proteases, alpha 2 macroglobulin and a range of lectins (e.g. MBL, ladderlectin and the pentraxins such as serum amyloid protein P), among other factors. *Y. ruckeri* has been shown to bind ladderlectin from trout serum in relatively low amounts (Young *et al.*, 2007) and it is not evident how this particular lectin acts defensively in this species as involvement in agglutination, direct opsonization or complement fixation has yet to be demonstrated. Resistance to lysozyme has not been demonstrated in *Y. ruckeri*, but the

presence of a peptidoglycan deacetylase is noteworthy. This enzyme alters the peptidoglycan backbone in lactic acid bacteria, including streptococci, making them resistant to host lysozyme activity (Vollmer and Tomasz, 2000, 2002). Moreover, it is essential for virulence of *S. pneumoniae* (Davis *et al.*, 2008*)* and has been identified as a virulence factor for *S. pyogenes* in a zebrafish (*Danio rerio*) infection model (Miller and Neely, 2004). While only the gene and a deduced amino acid sequence have been listed, and it may be that the encoded protein is a different type of deacetylase from the streptococcal virulence factor, this is an area that could be investigated further in *Y. ruckeri* resistance to innate immunity.

Most bacteria ultimately are removed and/or destroyed by macrophages. Thus, in order to establish infection, avoiding phagocytosis, or resisting or subverting the antimicrobial processes therein, is critical for most pathogens. One of the most thoroughly characterized of the virulence mechanisms of the plague bacterium *Y. pestis* and other *Yersinia* spp. is the plasmid-encoded Yop (*Yersinia* outer proteins) system (Trosky *et al.*, 2008). This plasmid encodes a Type III secretion system (TTSS) that delivers several effector proteins, also encoded on the plasmid, into the host cells targeting the actin system to cripple phagocytosis. They also target key signalling pathways in the immune attack, including MAPK kinases and IKB kinase-ß, reducing respiratory burst and promoting apoptosis (Trosky *et al.*, 2008). Although the pYV virulence plasmid is absent from *Y. ruckeri*, genes encoding a TTSS have been found in *Y. ruckeri*, but associated effector proteins have not yet been reported (Gunasena *et al.*, 2003). Initially, the Type III ATPase gene, *ysaN*, associated with TTSS was identified using degenerate PCR. Subsequently, the organization of the surrounding operon was determined and high homology was found between the *ysa* operon in *Y. ruckeri* and a cluster of genes encoding the *ysa* TTSS in *Y. enterocolitica* (Gunasena *et al.*, 2003). A role for this TTSS in *Y. ruckeri* has yet to be established.

As mentioned above, in the human pathogenic *Yersinia* spp. (*Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*), virulence is associated with a 40–50 MDa plasmid (Skurnik, 1985), which encodes the TTSS and effector Yop proteins that subvert phagocytic clearance of the pathogens (Trosky *et al.*, 2008). The association of plasmids with impaired phagocytic function has also been made in *Y. ruckeri*, in which a 70–88 kb plasmid (carried in many isolates of serovar I) decreased the chemiluminescent response of striped bass macrophages (De Grandis and Stevenson, 1982; Stave *et al.*, 1987). However, this plasmid was not homologous with virulence plasmids from other *Yersinia* spp. (Stave *et al.*, 1987). Serovar I isolates of *Y. ruckeri* harbouring a 70 MDa plasmid depleted the CL response of striped bass (*Morone saxatilis*) macrophages, although the factors expressed by the plasmid were not determined. Furthermore, one of the serovar II isolates, which did not carry the plasmid, also decreased the CL response of striped bass macrophages (Stave *et al.*, 1987). The decreased CL response may reflect the method used: chemiluminescent assays detect reactive oxygen species (ROS) and these may be quenched by bacterial enzymes such as superoxide dismutase (SOD) and catalase. Indeed, all serovar I isolates analysed contained at least one superoxide dismutase enzyme, depending on the growth conditions (A.C. Barnes, unpublished results). These enzymes may in themselves be important virulence factors, increasing resistance to phagocytic killing. It remains to be determined whether they are plasmid or chromosomally encoded.

Recently, the pYR1 plasmid has been described in *Y. ruckeri*, which carries several antibiotic resistance determinants. More interestingly, it also encodes a type IV conjugative transfer system (Welch *et al.*, 2007). *Y. ruckeri* also contains a chromosomally encoded type IV transfer system (*traH-N* operon) that is associated with virulence, as insertional mutants have attenuated virulence (Mendez *et al.*, 2009). The role of the type IV system in virulence of *Y. ruckeri* has not yet been explored fully, but it may be a requirement for intracellular survival of this facultative intracellular pathogen (Mendez *et al.*, 2009). The *tra* operon appears to be

temperature regulated with elevated expression at 18°C, in common with both Yrp1 protease, the YhlA haemolysin and the ruckerbactin system, suggesting temperature is a major determinant for virulence in *Y. ruckeri* (Fernandez *et al.*, 2003, 2004, 2007b; Mendez *et al.*, 2009).

Immunity

Although *Y. ruckeri* O-antigen has been used as a model antigen for the study of the kinetics of immune responses in rainbow trout (Anderson *et al.*, 1979a,b,c,d) and carp (Lamers and Pilarczyk, 1982), little is known of the key antigenic components of *Y. ruckeri* to which the fish responds during infection or following vaccination. The kinetics of the antibody response to O-antigen has been documented in rainbow trout with peak numbers of antibody-producing cells detected in the spleen 14 days after antigen administration by flush. The titres of circulating antibody increase to a maximum after about 28 days, with the onset of detection coinciding with maximum numbers of antibody-producing cells (Anderson *et al.*, 1979a). Immunization of rainbow trout, using commercial *Y. ruckeri* bacterin, also demonstrated a significant secondary response to antigen injected intraperitoneally 146 days after first exposure (Cossarini-Dunier, 1986b). While the development of an anamnestic immune response to *Y. ruckeri* in rainbow trout may be important, there is a lack of correlation between antibody titre and protection. Although humoral agglutinins were detected in brook trout 24 h after passive immunization by intraperitoneal injection of immune sera, these antibodies conferred no resistance to disease (Cipriano and Ruppenthal, 1987). Furthermore, protection in fry could not be achieved by passive transfer of antibodies maternally (Lillehaug *et al.*, 1996). Broodstock females were vaccinated repeatedly by injection and an increase in antibody titre, determined by ELISA and agglutination, was obtained against serovars I and II in the serum of vaccinated fish. Low levels were also detected in eggs and fry 1 week posthatching, although mortalities were not reduced in the offspring of vaccinated mothers compared with unvaccinated controls (Lillehaug *et al.*, 1996). This suggests that other immune mechanisms, such as a localized or cell-mediated response, must be involved in protection against *Y. ruckeri* infection. Cell-mediated response to *Y. ruckeri* has been less well studied. Jones *et al.* (1993) reported that killed cells of serovar I and serovar II strains stimulated proliferation of naive peripheral-blood leucocytes, and that the two strains were similarly mitogenic. However, the study concluded that this was irrespective of the immune status of the fish (Jones *et al.*, 1993). Antibodies produced by rainbow trout appear to be serotype-specific (Cipriano and Ruppenthal, 1987), as antibodies against serovar I do not recognize LPS in immunoblot, while those raised against 0:2/serovar II do bind LPS (Stevenson *et al.*, 1993). Additionally, a significantly higher dose of O-antigen from serovar I is required to elicit a response in rainbow trout than with O-antigen from serovar II (Anderson *et al.*, 1979c). In spite of the serological differences between *Y. ruckeri* serovars, there are numerous reports of cross-protection. Cipriano and Ruppenthal (1987) reported that, although immunization by intraperitoneal injection with bacterins against *Y. ruckeri* resulted in circulating antibody only to the specific serovar used in the bacterin, brook trout immunized in such a manner were protected during experimental challenge with both serovars I and II. Furthermore, a bacterin prepared from an avirulent serovar II isolate performed equally well compared with bacterins produced from virulent isolates (Cipriano and Ruppenthal, 1987). The antigens responsible for cross-protection have not yet been clearly identified; however, native flagellar antigens seem to be antigenically common among motile serogroups (Stevenson, 1997) and the Yrp1 protease confers protective immunity and appears to be conserved across diverse serotypes (Fernandez *et al.*, 2003).

Exploitation of molecular tools to investigate the immune response of trout to *Y. ruckeri* has provided key insights into factors that are important following both

vaccination and natural infection (Raida and Buchmann, 2008a,b, 2009). Measuring innate immune responses to primary and secondary infection by *Y. ruckeri* using quantitative real-time PCR (qRT-PCR) suggested activation of pathways via Toll-like receptors. This results in high upregulation of genes expressing acute phase proteins including the pentraxin serum amyloid protein A and pro-inflammatory cytokines interleukin 1 (IL-1b), IL-6 and tumour necrosis factor alpha (TNFa). Many complement factors are downregulated, including the critical opsonin C3. Secondary infection results in a lower response, but also in lower infection loads in the liver, with the authors suggesting onset of adaptive immunity may reduce the infection by reducing the innate response to secondary infections. The same authors also monitored levels of *Y. ruckeri* in the spleen of infected rainbow trout during primary and secondary infections (Raida and Buchmann, 2008a). They quantified expression of cytokine and receptor genes and found that upregulation of cytokines and T-cell receptors was high during primary infection and associated with high levels of *Y. ruckeri* in the spleen. However, on re-infection, the cytokine and T-cell receptor response was low and associated with much lower levels of bacteria in the spleen, from which the authors inferred adaptive immunity had developed in spite of the lack of upregulation of MHCII or IgT genes during either infection (Raida and Buchmann, 2008a). It appears that T-cell activation is critical for immunity to develop.

Using similar assays, Raida and Buchmann (2008b) also investigated the response to vaccination in trout. Following immersion vaccination of trout with a *Y. ruckeri* bacterin, protected fish upregulated expression of key innate immune genes including interferon gamma (IFN-g), TNFa, IL-6, IL-10, transforming growth factor beta (TGFb), T-cell receptor, CD8a, CD4, C5a receptor and immunoglobulin T (IgT). Interestingly, no protection of naive fish was conferred when plasma from immune animals was transferred passively, strongly suggesting that cellular immune factors, and particularly CD8 T-cells, may be critical for protection against infection by *Y. ruckeri*.

The generally successful vaccination of salmonids against ERM using *Y. ruckeri* bacterins based on serovar I and the numerous studies reporting cross-protection have perhaps led to a degree of complacency and a general consideration that other serovars are unnecessary in commercial vaccines (McCarthy and Johnson, 1982). Recent emergence of new biogroups (Austin *et al.*, 2003), coupled with past experience in the Norwegian industry (Erdal, 1989), would caution against such assumptions.

Future Directions

There has been substantial research progress in some areas since the previous edition of this chapter. One area in which there has been no progress, and which consequently still remains confusing, is intraspecific variability. The disagreement between different serotyping schemes and methodologies, coupled with the lack of broadly available common reagents, prevents any meaningful comparison of types between laboratories and makes characterization of new and emerging strains difficult. Multilocus sequence typing (MLST) based on sequencing defined fragments (generally 500–800 bp) of 7–8 housekeeping genes is a means by which strains may be typed and compared easily and accurately over the Internet (Maiden *et al.*, 1998). We have used this technique recently to pinpoint *S. iniae* isolates to specific countries, and have used a separate set of genes to track evolution on farms (Nawawi, 2010; Baiano and Barnes, unpublished). Careful selection of suitable genes with sufficient discriminatory variability is long overdue for *Y. ruckeri* and the MLST technique has great potential for providing a universal means of typing this species and tracking its evolutionary development and epidemiological spread.

In the first edition of this book, we commented that our understanding of the

virulence factors associated with *Y. ruckeri* was extremely limited based on a dearth of published information. In the intervening decade, this deficit has been addressed with some very elegant molecular and immunological studies of both pathogen and host. Work on the pathogen using *in vivo* expression technology and signature-tagged mutagenesis, followed up by studies using knockout mutants, has identified a number of key genes associated with virulence and has indicated that multiple factors are involved. Similarly, recent work analysing the expression of immune genes during primary and secondary infection, and following vaccination, has provided key insights into what protective mechanisms may be critical in fish. It is now time to bring these two fields of study together in a collaborative approach, perhaps investigating immune response to vaccines prepared from or challenged by the most interesting knockout mutants. The role of the flagellar antigens was mentioned briefly in the previous edition of this chapter. Their importance in virulence, and particularly in protective immunity, is now more apparent than ever, evidenced by the evolution of non-motile strains driven by vaccination.

At the time of the previous edition of this chapter, I surmised that the lack of research on virulence in this pathogen could be attributed to the early success of the vaccination programmes effectively abrogating the need for further research funding from the vaccine industry. With the emergence of new biotypes from vaccinated fish, additional funding in the areas suggested may prove a wise investment.

Acknowledgements

The first edition of this chapter in the first edition of this book was co-written by friend and former colleague, Mike Horne. Much of his original wisdom and historical insight is retained in the current revision, as it is as relevant today as it was 10 years ago. Thanks also to Dr Justice Baiano, an expert on bacterial 16S rRNA gene phylogenetics and a wizard with the Arb program.

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14 *Edwardsiella* Septicaemias

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Introduction

The genus *Edwardsiella* includes two species of bacteria that cause major diseases in fish: *E. tarda* (Ewing *et al*., 1965) infects fish and other animals and *E. ictaluri* (Hawke, 1979; Hawke *et al*., 1981) infects primarily fish. A third species, *E. hoshinae* (Grimont *et al*., 1980), infects birds and reptiles. *E. tarda* produces the disease commonly known as 'fish gangrene', 'emphysematous putrefactive disease of catfish' or 'red disease of eels', hereafter known in this text as *Edwardsiella* septicaemia (ES), and *E. ictaluri* causes 'enteric septicaemia of catfish' (ESC). Because *E. tarda* and *E. ictaluri* produce distinctively different diseases, they are discussed separately.

Edwardsiella Septicaemia (*Edwardsiella tarda*)

E. tarda infects invertebrates, freshwater and marine fish, reptiles and amphibians, avians and mammals throughout the world (Plumb, 1999a). *Edwardsiella* septicaemia is a serious systemic bacterial infection of cultured channel catfish (*Ictalurus punctatus*) (Meyer and Bullock, 1973) in the USA and of Asian catfish (*Clarias batrachus*) (Sahoo *et al*., 1998) and Indian major carp (Cyprinidae) (Sae-Oui *et al*., 1984; Swain and Nayak, 2003). In Japan and Taiwan it causes a serious infection, 'red disease' of cultured Japanese eel (*Anguilla japonica*) (Egusa, 1976; Liu and Tsai, 1980) and Japanese flounder (*Paralichthys olivaceus*) (Nakatsugawa, 1983). *E. tarda* causes substantial mortality in other species of eels, Australian eel (*A. reinhardti*) and European eel (*A. anguilla*), and in flatfish, turbot (*Scophthalmus maximus*), throughout Australia and Europe. In addition, *E. tarda* sporadically produces infection in a variety of other fish species throughout the world. *Edwardsiella* septicaemia in fish is characterized by necrotic abscesses in the muscle that emit a putrid odour when incised. The skin covering muscle abscesses can be whitish or show petechia. Mortality of infected fish may be acute but is often chronic, depending on the degree of stressful environmental conditions under which *E. tarda* infections occur.

The disease agent

Current classification

Isolates used to establish the genus *Edwardsiella*, described by Sakazaki and Murata

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(1962), were originally from human faeces in the USA and simultaneously from snakes in Japan in 1959 (Ewing *et al.*, 1965; Sakazaki, 1967; Sakazaki and Tamura, 1991). Hoshinae (1962) isolated an enteric bacterium from eels in Japan, which he named *Paracolobacterium anguillimortiferum*; however, the biochemical and biophysical characteristics of this organism were identical to those of *E. tarda* (ATCC, 15947), which held taxonomic precedence (Ewing *et al.*, 1965). Since no cultures of *P. anguillimortiferum* are available (Egusa, 1976), and to prevent confusion, *E. tarda* is the specific epithet (Farmer and McWhorter, 1984). Species of *Edwardsiella*, a member of the Enterobacteriaceae, are small, straight rods of about 1 μm in diameter and 2–3 μm in length (Farmer and McWhorter, 1984). They are Gram-negative, usually motile, with peritrichous flagella, and are facultatively anaerobic.

Biology

E. tarda commonly resides in the intestine of fish and other aquatic animals and may be found in many environments where a host is not required for survival, including sediments. It is present in both fresh water and marine water (Fourquet *et al.*, 1975; Ishihara and Kusuda, 1982; Chowdhury and Wakabayashi, 1990), mud (White *et al.*, 1973; Wyatt *et al.*, 1979) and fouling material in nets (Kanai *et al.*, 1988). *E. tarda* survives in water from 14 to 45°C, with highest survival at 30–37°C, at pH 4.0–10.0 (optimum 7.5–8.0) and at 0–4% sodium chloride (NaCl) (optimum 0.5–1.0%) (Ishihara and Kusuda, 1982). The organism can be isolated for over 76 days after inoculating sterile pond water (20°C), indicating that it can survive for long periods in the environment (Plumb, 1999a). *E. tarda* may also persist in fresh and marine environments for extended periods in a viable but nonculturable (VBNC) form (Sakai *et al.*, 1994). Experimental induction of a VBNC state under starvation conditions at low temperatures (4°C) has been demonstrated (Du *et al.*, 2007).

Phenotypic and antigenic characteristics

Important characteristics of *E. tarda* of presumptive diagnostic value are motility, indole production, cytochrome oxidase reaction, reaction on triple sugar iron (TSI) agar, citrate and methyl red media and salt and temperature tolerance. *E. tarda* is a typical member of the Enterobacteriaceae; it is a Gram-negative motile rod that is catalase-positive, oxidase-negative and glucose-fermentative, reduces nitrate to nitrite, is lactose-negative and indole-positive and produces an alkaline slant, acid butt and hydrogen sulfide (H_2S) on TSI agar (Shotts and Teska, 1989) (Table 14.1).

Waltman *et al.* (1986a) compared 116 *E. tarda* isolates from the USA and Taiwan and found little variation in their biochemical and biophysical characteristics, although the isolates from Taiwan differed slightly from those in the USA. Farmer and McWhorter (1984) listed a wild-type *E. tarda* and a second biogroup 1 by biochemical and biophysical characteristics. Strains of *E. tarda* which are negative for D-mannitol, sucrose and L-arabinose are more common than biogroup 1 and therefore are designated '*E. tarda* wild type'. Striped bass (*Morone saxatilis*) wild-type isolates, characterized by gas chromatography of fatty acid methyl esters (FAME), had a FAME profile typical of *E. tarda*, the major peak being hexadecanoic acid (16:0), followed in descending amounts by saturated and unsaturated fatty acids and trace amounts of decanoate and octadeconoate (Baya *et al.*, 1997). Panangala *et al.* (2006b), in a comparison of nine *E. tarda* isolates from disparate disease outbreaks, found 95% similarity in their FAME profile and 30% similarity in their protein profiles. Using the Minitek numerical identification system, Taylor *et al.* (1995) identified *E. tarda* correctly 100% of the time compared with 83% positive identification with the API 20E system.

Serotypes

E. tarda may be divided into serological groups based on the O-agglutination test.

Table 14.1. Biochemical and biophysical characteristics of *Edwardsiella tarda*, *E. ictaluri* and *E. hoshinae* (Grimont *et al.*, 1980; Hawke *et al.*, 1981; Farmer and McWhorter, 1984; Waltman *et al.*, 1986a,b; Plumb and Vinitnantharat, 1989; OIE, 2006).

	E. tarda			
Characteristic	Wild type	Biogroup 1	*E. ictaluri*	*E. hoshinae*
Motility at:				
25°C[a]	+	+	+	+
37°C[a]	+	+	–	+
Growth at 40°C	+		–	+
Tolerance to NaCl				
1.5%[a]	+		+	+
4.0%[a]	+		–	–
Cytochrome oxidase[a]	–		–	–
Indole[a]	+	+	–	+
Methyl red[a]	+		–	+
Citrate (Christensen's)[a]	+	+	–	+
H_2S production:				
Triple sugar iron[a]	+	–	–	–
Peptone iron sugar	+		–	+
Lysine decarboxylase	+		+	+
Ornithine decarboxylase	+		+	+
Malonate utilization	–	–	–	+
Gas from glucose	+		+	+
Acid production from:				
D-mannose, maltose	+		+	+
D-mannitol, sucrose	–	+	–	+
Trehalose	–	–	–	+
L-Arabinose	–	+	–	+
Jordan's tartrate	+		–	–
Nitrite from nitrate	+		+	+
Tetrathionate reductase	+			+
Edwardsiella isolation media (EIM)	Black centres		Green	Unknown
Mol % G + C of DNA	55–58		56–57	53

+, Positive for 90–100% of isolates; –, negative for 90–100% of isolates; ±, mixed reactions; G+C, guanine plus cytosine; DNA, deoxyribonucleic acid. [a]Key characteristics for presumptive identification. All isolates of *E. tarda*, *E. ictaluri* and *E. hoshinae* tested are negative for Voges–Proskauer, Simmons citrate, urea, phenlyalanine deaminase, arginine dihydrolase, gelatin hydrolysis, growth in KCN; acid production from glycerol, salicin, adonitol, D-arabilol, cellobiose, dulcitol, erythritol, lactose, inositol, melibiose, α-methyl-D-glucoside, raffinose, L-rhamnose, D-xylose and mucate; aesculin hydrolysis, acetate utilization, deoxyribonuclease, lipase, 3-galactosidase (*o*-nitrophenol-β-D-galactopyranoside, ONPG), pectate hydrolysis, pigment production and tyrosine clearing.

Park *et al.* (1983) used this test to identify serotypes of 61% (270) of 445 *E. tarda* isolates from kidney of infected eel, rectum of eels and other fish, and water and sediments from eel ponds which were classified into four serotypes (A, B, C and D). Of the isolates from eel kidney, 72% were serotype A. Rashid *et al.* (1994b) also determined that 28 strains of *E. tarda* from diseased flounder were identical to serotype A. A number of *E. tarda* serotypes have been identified based on somatic (O) and flagellar (H) antigens (Tamura *et al.*, 1988). The discovery of two proteins belonging to the type III secretion system, flagellin and SseB proteins, enabled a distinction between virulent and avirluent isolates of *E. tarda* (Tan *et al.*, 2002, 2005). In an effort to distinguish whether *E. tarda* isolates from fish were the same as those from human infections, Nucci

et al. (2002) used polymerase chain reaction (PCR) to compare genes associated with various pathogenic traits and found that few differences could be distinguished. However, a random amplified polymorphic DNA (RAPD) test distinguished, with few exceptions, recognizable genetic differences between isolates of human and fish origin.

Transmission

E. tarda is transmitted through the water from an infected source (carrier animal faeces, water or mud) to susceptible fish. Most probably, fish become naturally infected via injuries to the skin epithelium or through the intestine. Fish can be infected experimentally with *E. tarda* by intraperitoneal (i.p.) and intramuscular (i.m.) injection, stomach gavage or immersion in a bath containing the pathogen. A disease identical to natural infections in tilapia was reproduced by i.m. injection by Miyashita (1984). However, infection is not guaranteed simply by introducing the fish to the pathogen. Huang and Liu (1986) killed 100% of i.p. injected eels when *E. tarda* was mixed with *A. hydrophila*, but waterborne exposure to the same mixture failed to induce mortality unless sublethal concentrations of nitrogenous compounds were present. Miyazaki *et al.* (1992) injured the intestine with hydrogen peroxide prior to introducing *E. tarda* into the lumen via a silicon tube. This procedure resulted in death 5–23 days later and the infected fish developed pathological lesions in kidneys and livers that were nearly identical to those observed in natural infections. Similarly, Darwish *et al.* (2000) and Pressley *et al.* (2005) produced ES by first injuring the skin mechanically prior to immersion in a solution containing *E. tarda*. Wiedenmayer *et al.* (2006), however, were able to infect channel catfish following immersion exposure without prior mechanical skin injury. Using a green fluorescent protein-tagged *E. tarda*, the sites of entry of virulent *E. tarda* were demonstrated to be the gastrointestinal tract, gills and the body surface of fish (Ling *et al.*, 2000, 2001).

Transmission of *E. tarda* in catfish, eels and most other fish species appears to be enhanced at water temperatures from 20 to 30°C. Japanese (olive) flounder (*P. olivaceus*) were most susceptible at 20–25°C by i.m. injection in which a median lethal dose (LD_{50}) of 7.1×10^1 colony-forming units (cfu)/fish was established (Mekuchi *et al.*, 1995a). This compared with an LD_{50} of 1.7×10^2 cfu for i.p. injection, 3.6×10^6 cfu/ml for immersion and 1.3×10^6 cfu/fish oral exposure at the same temperatures. In experimental studies at 22°C, gilthead sea bream (*Sparus aurata*), turbot and striped bass had LD_{50}s of 3, 4 and 7×10^5 cfu, respectively, following injection (Baya *et al.*, 1997).

Higher cumulative percent mortality (>90%) in Japanese flounder was observed when fish were co-infected with marine birnavirus and *E. tarda* or *Vibrio harveyi* by i.p. injection, whereas birnavirus alone did not cause mortality (Oh *et al.*, 2006). Ciliated protozoans are one possible route of *E. tarda* transmission. Plumb (1999b) has observed tilapia with a heavy or moderate *Trichodina* infestation become clinically infected with *E. tarda*. King and Shotts (1988) demonstrated that co-culture of *E. tarda* or *Aeromonas salmonicida* with *Tetrahymena pyriformis* resulted in multiplication of the digested and intracellular bacteria within the protozoan. The growth of the bacteria within the protozoan may have important implications in transmission of *E. tarda* by a protozoan vector.

Host range and geographical distribution

E. tarda infects a wide variety of fish (Table 14.2). At least 40 species of fish from more than 20 families are known to have been infected and probably all species are susceptible under certain conditions.

Most diseases associated with *E. tarda* occur in cultured fish in either fresh or marine waters. The most prominent hosts are eels: Japanese eel (*A. japonica*) in Japan (Egusa, 1976) and European eel (*A. anguilla*) in Europe (Taksdal *et al.*, 1989; Alcaide *et al.*, 2006). *E. tarda* has also been isolated from wild Australian eel (*A. reinhardti*) in

Table 14.2. Species of fish, habitat: aquarium (A), cultured (C), wild (W) or experimental (E), continents and countries from which *Edwardsiella tarda* has been reported. NR, not reported.

Fish species	Habitat	Continent	Country	Reference(s)
Angelfish (*Pterophyllum scalare*)	A	Europe	Belgium	Vandepitte *et al.* (1983)
Atlantic salmon (*Salmo salar*)	W	N. America	Canada	Martin (1984)
Australian eel (*Anguilla reinhardti*)	W, E	Australia	Australia	Eaves *et al.* (1990)
Black skirt tetra (*Gymnocorymbus temetzi*)	A	NR	NR	Bullock and McCraren (1989)
Three-spot/blue gourami (*Trichogaster trichopterus*)	A, E	Asia	Singapore	Dixon and Contreras (1992); Ling *et al.* (2001)
Barramundi (Asian sea bass) (*Lates calcarifer*)	C	Australia	Australia	Iveson (1971)
Blue tilapia (*Tilapia aureus*)	E	Asia	Taiwan	Wang and Wang (1997)
Brook trout (*Salvelinus fontinalis*)	C	N. America	Canada	Uhland *et al.* (2000)
Brown trout (*Salmo trutta trutta*)	E	N. America	USA	Meyer and Bullock (1973)
Brown bullhead (*Ictalurus nebulosus*)	NR	NR	NR	Bullock and McCraren (1989)
Carp (*Labeo rohita*)	C	Asia	India	Swain and Nayak (2003)
Channel catfish (*Ictalurus punctatus*)	C	N. America	USA	Meyer and Bullock (1973)
Chinook salmon (*Oncorhynchus tshawytscha*)	C, W	N. America	USA	Amandi *et al.* (1982)
Climbing perch (*Anabas testudineus*)	C	Asia	India	Sahoo *et al.* (2000)
Coloured (common) carp (*Cyprinus carpio*)	C	Asia	India	Sae-Oui *et al.* (1984)
Common (Indian) carp (*Catla catla*)	C	Asia	India	Swain and Nayak (2003)
Crimson sea bream (*Evynnis japonica*)	C	Asia	Japan	Kusuda *et al.* (1977)
Emerald shiner (*Notropis atherinoides*)	NR	NR	NR	Bullock and McCraren (1989)
European eel (*Anguilla anguilla*)	C/W	Europe	Norway/Spain	Taksdal *et al.* (1989); Alcaide *et al.* (2006)
European sea bass (*Dicentrarchus labrax*)	C	Europe	Spain	Blanch *et al.* (1990)
Gilthead sea bream (*Sparus aurata*)	E	N. America	USA	Baya *et al.* (1997)
Glowlight tetra (*Hemigrammus erythrozonus*)	A	Asia	Singapore	Chua (1996)
Goldfish (*Carassius auratus*)	NR	NR	NR	Bullock and McCraren (1989)
Grass carp (*Ctenopharyngodon idella*)	E	Asia	Taiwan	Bullock and McCraren (1989); Wang and Wang (1997)
Japanese eel (*Anguilla japonica*)	C	Asia	Japan	Hoshinae (1962); Egusa (1976)
	C		Korea	Kokuska (1973)
	C		Taiwan	Liu and Tsai (1980)

Japanese (olive) flounder (*Paralichthys olivaceus*)	C	Asia	Japan	Nakatsugawa (1983)
Banded knifefish (*Gymnotus carapo*)	C	Australia	Sydney Zoo	Reddacliff *et al*. (1996)
Largemouth bass (*Micropterus salmoides*)	W	N. America	USA	White *et al*. (1973); Francis-Floyd *et al*. (1993)
Mullet (*Mugil cephalus*)	W	Asia	Japan	Kusuda *et al*. (1976)
Malawi blue cichlid (*Maylandia zebra*)	A	Asia	Singapore	Chua (1996)
Mozambique tilapia (*Tilapia mossambicus*)	C	Asia	Japan	Kaige *et al*. (1986)
Nile tilapia (*Oreochromis niloticus*)	C	Asia	Japan	Aoki and Kitao (1981); Miyashita (1984)
	C	Africa	Egypt	Badran (1993); Saleh (2005)
	W	Africa	Zaire	Van Damme and Vandepitte (1980)
	W	Africa	Transvaal	Bragg (1988)
	C	Europe	Turkey	Benli and Yildiz (2004)
Oyster toadfish (*Opsanus tau*)	W, C	N. America	USA	Horenstein *et al*. (2004)
Rainbow trout (*Oncorhynchus mykiss*)	C, E	Australia	New S. Wales	Reddacliff *et al*. (1996)
Rosy barb (*Puntius conchonius*)	A	Asia	Singapore	Humphrey *et al*. (1986)
Red sea bream (*Pagrus major*)	C	Asia	Japan	Yasunaga *et al*. (1982); Choi (1991); Sano *et al*. (2001)
Red tilapia tetrahybrids	C	S. America	Venezuela	Clavijo *et al*. (2002)
Sand goby (*Oxyeleotris marmoratus*)	C	Asia	Thailand	Supamataya (1988)
Siamese fighting fish (*Betta splendens*)	A	Europe	Czechoslovakia	Vladick *et al*. (1983); Humphrey *et al*. (1986)
Spotted bonytongue (*Scleropages leichardu*)	A	Australia	Sydney Zoo	Reddacliff *et al*. (1996)
Spotted snakehead (*Channa punctata*)	C	Asia	India	Kumar *et al*. (2007)
Striped bass (*Morone saxatilis*)	W	N. America	USA	Herman and Bullock (1986); Baya *et al*. (1997)
Turbot (*Scophthalmus maximus*; *Psetta maxima*)	C, E	Europe	Spain	Nougayrede *et al*. (1994); Baya *et al*. (1997); Castro *et al*. (2006)
Yellowtail (*Seriola quinqueradiata*)	C	Asia	Japan	Yasunaga *et al*. (1982)
Walking catfish (*Clarias batrachus*)	C	Asia	India	Sahoo *et al*. (1998)
Wels catfish (*Silurus glanis*)	E	Europe	France	Caruso *et al*. (2002)
Zebrafish (*Danio reiro*)	A, E	N. America	USA	Pressley *et al*. (2005)

Australia (Eaves *et al.*, 1990). Channel catfish are prominent hosts in the USA (Meyer and Bullock, 1973), although epidemics in wild striped bass (Herman and Bullock, 1986; Baya *et al.*, 1997) and largemouth bass (*Micropterus salmoides*) (White *et al.*, 1973; Francis-Floyd *et al.*, 1993) have been reported. Many other cultured fish species are also significantly affected: for example, Japanese flounder (Nakatsugawa, 1983), yellowtail (*Seriola quinqueradiata*) (Yasunaga *et al.*, 1982), crimson sea bream (*Evynnis japonica*) (Kusuda *et al.*, 1977) and red sea bream (*Pagrus major*) (Yasunaga *et al.*, 1982; Choi, 1991) in Japan, a variety of cultured Cyprinideae (minnows and carp), in particular Indian major carp (*Catla catla* and *Lobeo rohita*) (Swain and Nayak, 2003) and common carp (*Cyprinus carpio*) (Sae-Oui *et al.*, 1984), walking catfish (*C. batrachus*), climbing perch (*Anabas testudineus*) (Sahoo *et al.*, 1998) and spotted snakehead (*Channa punctata*) (Kumar *et al.*, 2007) in India and tilapia (*Oreochromis* spp.) in Africa and Asia (Van Damme and Vandepitte, 1980; Aoki and Kitao, 1981; Miyashita, 1984; Badran, 1993; Saleh, 2005). Significant losses in cultured marine species such as turbot (Nougayrede *et al.*, 1994; Castro *et al.*, 2006) and European sea bass (*Dicentrarchus labrax*) (Blanch *et al.*, 1990) have also been reported. Isolation of *E. tarda* has occurred from numerous tropical ornamentals and/or experimental aquarium fish (Vandepitte *et al.*, 1983; Humphrey *et al.*, 1986; Bullock and McCraren, 1989; Dixon and Contreras, 1992; Chua, 1996; Reddacliff *et al.*, 1996; Pressley *et al.*, 2005). *E. tarda* generally causes disease in warmwater fish only, but clinical infections have also occurred in migrating Chinook salmon (*Oncorhynchus tshawytscha*) in the Rogue River, Oregon, USA (Amandi *et al.*, 1982), and spawning Atlantic salmon (*Salmo salar*) in Nova Scotia, Canada (Martin, 1984), as well as in farmed rainbow trout (*O. mykiss*) in Australia (Reddacliff *et al.*, 1996) and brook trout (*Salvelinus fontinalis*) broodstock in Quebec, Canada (Uhland *et al.*, 2000). The list of countries in which *E. tarda* has been found in fish is extensive but includes predominantly cultured fish from the USA, Japan, India, Taiwan, Korea, Spain, Egypt and Venezuela, and wild fish from Australia, Canada, USA and Third World countries (Table 14.2).

E. tarda infections are not limited to fish. Other animals may be carriers and/or reservoirs of the pathogen. *E. tarda* is a ubiquitous organism, having been isolated from animals or the environment from more than 30 countries of most continents (Plumb, 1999a). The list of countries in which *E. tarda* has been found in hosts other than fish and environmental sources is extensive but includes predominantly the USA, Japan, India, the Philippines and many Third World countries (Plumb and Evans, 2006) (Table 14.3). The bacterium has been isolated from invertebrates (Sasaki and Aita, 1975; Bartlett and Trust, 1976; Wyatt *et al.*, 1979), amphibians (frogs and toads) (Kourany *et al.*, 1977; Kumar and Sharma, 1978; Wyatt *et al.*, 1979), reptiles (snakes, turtles, lizards, crocodiles and alligators) (Wallace *et al.*, 1966; Nagel *et al.*, 1982; Sugita and Deguchi, 1983), birds (White *et al.*, 1969, 1973; Berg and Anderson, 1972; Forrester *et al.*, 1976; Taylor, 1992), cattle and swine (Ewing *et al.*, 1965; Arambulo *et al.*, 1967; Arambulo and Westerlund, 1968; Elazhary *et al.*, 1973; Owens *et al.*, 1974), dogs (Tacal and Menez, 1968), marsupials (How *et al.*, 1983), raccoons (White *et al.*, 1975), marine mammals (Wallace *et al.*, 1966; Frye and Herald, 1969; Forrester *et al.*, 1975; Coles *et al.*, 1978) and other warm-blooded animals (Van Damme and Vandepitte, 1984) (Table 14.3). In many instances, *E. tarda* is part of the normal intestinal microflora of aquatic animals (Wyatt *et al.*, 1979; Van Damme and Vandepitte, 1980; Kanai *et al.*, 1988). Bauwens *et al.* (1983) found *E. tarda* in the intestine of a variety of zoo animals, especially fish-eaters and water-loving species. Likewise, Van Damme and Vandepitte (1984) isolated *E. tarda* from numerous zoo species. *E. tarda* has also been associated with several different disease manifestations in humans (King and Adler, 1964; Jordan and Hadley, 1969; Bockemühl *et al.*, 1971; Van Damme and Vandepitte, 1980; Vandepitte *et al.*, 1983; Hargreaves and Lucey, 1990; Murphey *et al.*, 1992; Janda

and Abbott, 1993b; Ashford *et al.*, 1998; Blomkalns and Otten, 1999; Mizunoe *et al.*, 2006).

Economic, environmental and social importance of the disease

The economic impact of *E. tarda* on morbidity and mortality of Japanese flounder and red sea bream culture is severe (Igarashi and Iida, 2002), ranging from 5 to 30% and 5 to 70%, respectively (Pastor, 1981; Pavanelli *et al.*, 1998; Igarashi and Iida, 2002). While *E. tarda* infection occurs in a variety of cultured and wild fish populations, little or no information is available regarding its prevalence, incidence or chemotherapeutic costs to assess economic impact, primarily because of the lack of validated and widely used detection assays. An epidemiological and ecological investigation of *E. tarda* on two Japanese flounder farms in Japan revealed that culture water, sediment and fish were frequently positive for *E. tarda* (Rashid *et al.*, 1994a). *Edwardsiella* septicaemia is often a chronic problem that may increase not only mortality but also production costs, reduce feed conversion and delay harvest. Cumulative losses of fish due to *E. tarda* infection may be moderate to high, thus resulting in reduced availability of fish to the market and loss of revenue to the producer. This problem is exacerbated by the fact that the infection most often affects market-size fish, but it is not confined to this age or size group. Generally, *E. tarda* has a mild impact on cultured channel catfish; however, stressful environmental conditions can cause a nonclinical infection to progress into a clinical infection, resulting in significant mortality (Meyer and Bullock, 1973). *E. tarda* sometimes produces an undiagnosed subclinical infection in fish intended for human consumption, where it may create problems during processing (Meyer and Bullock, 1973). When subclinically infected fish are processed, the equipment becomes contaminated, which interrupts processing. Operations must then be shut down to disinfect all equipment and dispose of infected fish. Also, gulls with *E. tarda* in their faeces could contaminate fish processing plants along the coast (Berg and Anderson, 1972).

The impact of *E. tarda* in wild fish populations is unknown due to the absence of routine surveillance, monitoring and investigation of the effects of *E. tarda* infection. Epidemics in two commercially and recreationally valuable warmwater species, largemouth bass and striped bass, have occurred in the USA (Francis-Floyd *et al.*, 1993; Baya *et al.*, 1997). Seasonal mortality of largemouth bass may reduce the annual revenues of US$600 million, the reported value of this freshwater game fish indigenous to Florida lake systems (Hardin *et al.*, 1987). *E. tarda* has little economic impact on salmonids as it is not considered a common pathogen (Amandi *et al.*, 1982; Martin, 1984; Reddacliff *et al.*, 1996; Uhland *et al.*, 2000).

Only minor environmental impact results from *E. tarda* because this bacterium is common in the environment (Wyatt *et al.*, 1979; Rashid *et al.*, 1994a). Ecological changes resulting from its introduction have not been reported, perhaps due to the lack of cross-disciplinary studies. *E. tarda* has no recorded impact on biodiversity.

As a zoonotic pathogen, *E. tarda* may have a social impact since it can infect humans and other warm-blooded animals (Bockemühl *et al.*, 1971). Human infections have been contracted from infected fish during handling or by consuming uncooked contaminated fish (Plumb, 1999a). In humans, infections of the digestive tract can result from ingesting infected fish (Clarridge *et al.*, 1980; Noga, 2000). Infections in muscle tissue of healthy persons via puncture wounds by spines of infected catfish can also occur (Hargreaves and Lucey, 1990; Vartian and Septimus, 1990; Murphey *et al.*, 1992; Ashford *et al.*, 1998; Blomkalns and Otten, 1999). Janda and Abbott (1993a) cited additional human risk factors in *E. tarda* zoonoses including occupational and recreational exposure to aquatic environments or exotic animals and dietary preferences such as ingestion of raw fish. In addition, age (adults over 50 and children), geographical distribution (tropical and subtropical

Table 14.3. Non-fish hosts, environmental sources and countries from which *Edwardsiella tarda* and *E. ictaluri* have been isolated.

Hosts	Country	Reference	Hosts	Country	Reference
Mammals			Avians		
Humans	Australia	Ashford *et al.* (1998)	Bald eagle	USA	White *et al.* (1973)
	Belgium	Vandepitte *et al.* (1983)	Brown pelicans	USA	White *et al.* (1973)
	China	Zheng (1987)	Common loon	USA	White *et al.* (1973)
	Cuba	Rádovsky and Aldová (1965)	Crane	Zaire	Van Damme and Vandepitte (1984)
	India	Koshi and Lalitha (1976); Sharma (1979)	Duck	Zaire	Van Damme and Vandepitte (1984)
	Germany	Bockemühl *et al.* (1983)	Eagle	Zaire	Van Damme and Vandepitte (1984)
	Italy	Maserati *et al.* (1985)	Blue heron	USA	White *et al.* (1973)
	Israel	Sechter *et al.* (1983)	Gulls	USA	Berg and Anderson (1972); White *et al.* (1973)
	Japan	Sakazaki (1967); Mizunoe *et al.* (2006)	Little penguins	Australia	Reddacliff *et al.* (1996)
	Madagascar	Fourquet *et al.* (1975)	Hawk	Zaire	Van Damme and Vandepitte (1984)
	Malaysia	Gilman *et al.* (1971)	Ibis	Zaire	Van Damme and Vandepitte (1984)
	Mali	Vandepitte *et al.* (1980)	Ostrich	USA	White *et al.* (1969)
	Norway	Bergen *et al.* (1988)	Falcon		Reichel *et al.* (1974)
	Panama	Kourany *et al.* (1977)	Pigeon	France	Chamoiseau (1967)
	Spain	Martínez (1987)	Sandhill crane	USA	White *et al.* (1973); Forrester *et al.* (1976)
	Thailand	Bockemühl *et al.* (1971); Boonyaratpalin (1983)	Reptiles	Australia	How *et al.* (1983)
	USA	Ewing *et al.* (1965); Jordan and Hadley (1969); Clarridge *et al.* (1980)	Crocodiles	Australia	Iveson (1971)
			Alligators	USA	Wallace *et al.* (1966); White *et al.* (1973)

	USSR	Kalina (1980)
	Vietnam	Nguyen-Van-Ai *et al.* (1975)
	Zaire	Makula *et al.* (1973); Van Damme and Vandepitte (1980)
Cattle	Philippines	Arambulo and Westerlund (1968)
	Zaire	Van Damme and Vandepitte (1984)
Swine	Canada	Elazhary *et al.* (1973)
	Philippines	Arambulo *et al.* (1967); Arambulo and Westerlund (1968)
	USA	Ewing *et al.* (1965); Owens *et al.* (1974)
Dogs	Philippines	Tacal and Menez (1968)
Zoo mammals	Belgium	Bauwens *et al.* (1983)
	Zaire	Van Damme and Vandepitte (1984)
Wildlife		
Marsupials	Australia	How *et al.* (1983)
Raccoon	USA	White *et al.* (1975)
Marine mammals		
Porpoise	USA	Coles *et al.* (1978)
Manatee	USA	Frye and Herald (1969); Forrester *et al.* (1975)
Skink	USA	White *et al.* (1969)
Lizards	Germany	Roggendorf and Müller (1976)
	Nigeria	Gugnani *et al.* (1986)
	Singapore	Tan and Lim (1977)
Snakes	Australia	Iveson (1971)
	Chad	D'Empaire (1969)
	Japan	Sakazaki and Murata (1962); Sugita and Deguchi (1983)
	India	Sharma (1979)
	Germany	Müller (1972); Roggendorf and Müller (1976)
	Panama	Kourany *et al.* (1977)
	USA	Jackson *et al.* (1969a,b); Otis and Behler (1973); Wyatt *et al.* (1979)
Tortoise/turtles	Chad	D'Empaire (1969)
	Israel	Sechter *et al.* (1983)
Amphibians		
Toads and frogs	India	Kumar and Sharma (1978)
	Panama	Kourany *et al.* (1977)
	USA	Wyatt *et al.* (1979)
Invertebrates		

(*Continued*)

Table 14.3. *Continued*

Hosts	Country	Reference	Hosts	Country	Reference
Sea lions	USA	Wallace *et al.* (1966); Coles *et al.* (1978)	Sea urchins	Japan	Sasaki and Aita (1975)
Seal	Zaire	Van Damme and Vandepitte (1984)	Crayfish	USA	Wyatt *et al.* (1979)
Water	Bangladesh	Chowdhury and Wakabayashi (1990)	Snail	USA	Bartlett and Trust (1976)
	Israel	Sechter *et al.* (1983)	Sediment	Japan	Rashid *et al.* (1994a)
	Japan	Rashid *et al.* (1994a)			
	USA	White *et al.* (1973); Wyatt *et al.* (1979)			
E. ictaluri					
Blue heron	USA	Taylor (1992)	Great egret	USA	Taylor (1992)
Cormorants	USA	Taylor (1992)	Snowy egret	USA	Taylor (1992)

regions) and pre-existing liver disease and underlying iron overload states (cirrhosis, red cell sickling and leukaemia) may predispose humans to *E. tarda* infection (Bockemühl *et al.*, 1971; Janda and Abbott, 1993a). *E. tarda* is also an emergent bacteria for foodborne disease of humans (Stiles, 1989). Consequently, its potential impact on food production and safety must be considered to be significant. Bernoth (1991) reported that *E. tarda* might be pathogenic when transmitted from fish to other animals and their environments. Recently, *E. tarda* was demonstrated to be transmitted from the faeces of pigs to cultured tilapia on an integrated fish farm (Muratori *et al.*, 2000), suggesting that the high incidence of *E. tarda* isolation from external surfaces was a potential risk for food contamination. Due to the unappealing appearance of fish infected with *E. tarda*, a poor perception of fishery products from areas where infection has been reported may deter their purchase and consumption by humans for fear of sickness. Tourism may also be impacted as a result of human perception of an unclean environment.

Diagnostic methods

Clinical characteristics

Clinical signs of *E. tarda* infections vary between species of fish; therefore, they are generally of little use, except to indicate the likely presence of a bacterial infection. It is necessary to isolate bacteria from diseased fish, because clinical signs of *A. hydrophila*, *E. tarda*, *Pseudomonas anguillaseptica* and *V. anguillarum* and infections generally cannot otherwise be differentiated (Nishibuchi *et al.*, 1980).

Whether the infection is mild or severe, clinical signs of ES infection differ slightly between species of fish. In channel catfish, *E. tarda* initially produces small, 1–5 mm cutaneous lesions located dorsolaterally in the muscle (Meyer and Bullock, 1973). These small lesions progress to larger necrotic abscesses within the flank muscle or caudal peduncle, where they form obvious convex swollen areas and the skin loses its pigmentation (Fig. 14.1). Lesions in the muscle contain large amounts of necrotized tissue and emit a putrid odour when incised; hence the name, emphysematous putrefactive disease of catfish (Meyer and Bullock, 1973). As infection progresses, affected fish lose mobility of the caudal portion of the body and a generalized internal hyperaemia, similar to other bacterial septicaemias, is evident. The kidney, in particular, is enlarged and the liver is mottled; both organs are soft. Initially, it was believed that *E. tarda* caused disease in larger channel catfish only (i.e. over 0.4 kg). However, there seems to be no distinct size or age differential in susceptibility of any species of fish (Herman and Bullock, 1986). Japanese eels with acute *E. tarda* infection develop severe hyperaemia, with bloody congestion of fins (Fig. 14.2a), petechial or ecchymotic haemorrhage on various body surfaces, gas-filled pockets in the skin and large bloody necrotic lesions in the muscle (Fig. 14.2b). The anal region is swollen and hyperaemic. Internally, there is a general hyperaemia of the peritoneum; the liver is mottled, oedematous and abscessed.

A variety of clinical signs occur in other species of fish. For example, *E. tarda* infection causes exophthalmia and/or ocular opacity in tilapia, skin discoloration, haemorrhage and gas-filled cavities in the muscle, swollen spleen and kidney, as well as abscesses in internal organs (Kubota *et al.*, 1981; Badran, 1993; Plumb, 1997). Japanese flounder, naturally infected with *E. tarda*, develop ulcerative lesions and loss of skin, which expose underlying muscle, haemorrhage in fins, rectal protrusion and swelling of the spleen. Infected cage-cultured largemouth bass develop necrotic lesions on the caudal peduncle (see Fig. 14.1) (Plumb, 1999a). Striped bass exhibit many of these clinical signs, in addition to large amounts of sanguinous opaque mucoid fluid in the body cavity (Baya *et al.*, 1997). Consistent signs of disease in turbot include haemorrhage in the musculature, primarily in the head region, reddening around the mouth and at the base of fins, discoloration of the skin, abundant ascitic fluid in the abdominal cavity, liver discoloration, haemorrhagic

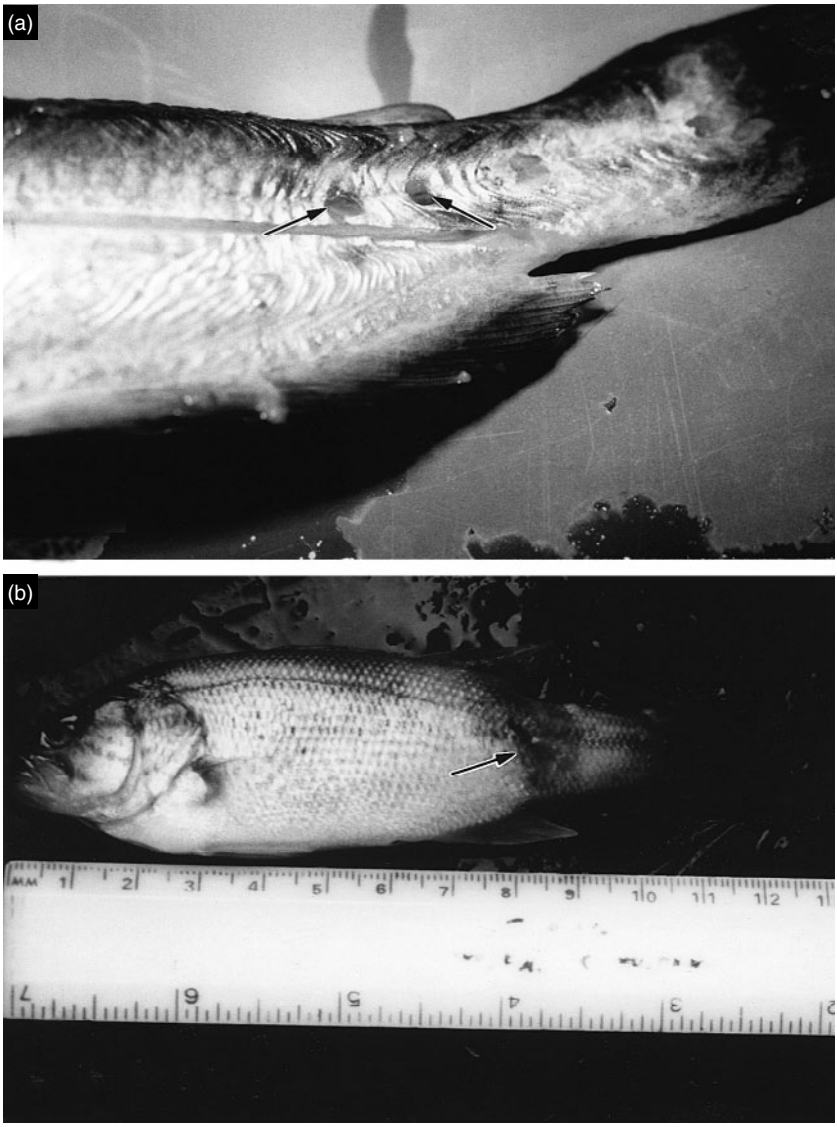


Fig. 14.1. (a) *Edwardsiella tarda* infection in channel catfish. The muscle tissue is necrotic, has lost its firmness and has open lesions (arrow) (photograph by Ahmed Darwish); (b) a juvenile largemouth bass with necrotic *E. tarda* lesion on the caudal peduncle (arrow).

kidney and abscesses in the kidney, spleen and liver (Nougayrede *et al.*, 1994; Castro *et al.*, 2006; Padrós *et al.*, 2006) .

In humans, the bacterium usually causes diarrhoea and gastroenteritis (King and Adler, 1964; Jordan and Hadley, 1969; Bockemühl *et al.*, 1971; Van Damme and Vandepitte, 1980), while extraintestinal infections may produce a typhoid-like illness, peritonitis with sepsis and cellulitis (Fields *et al.*, 1967). Occasionally, *E. tarda*-induced abscesses have been seen in the liver (Zighelboim *et al.*, 1992). Several other clinical conditions in humans have been

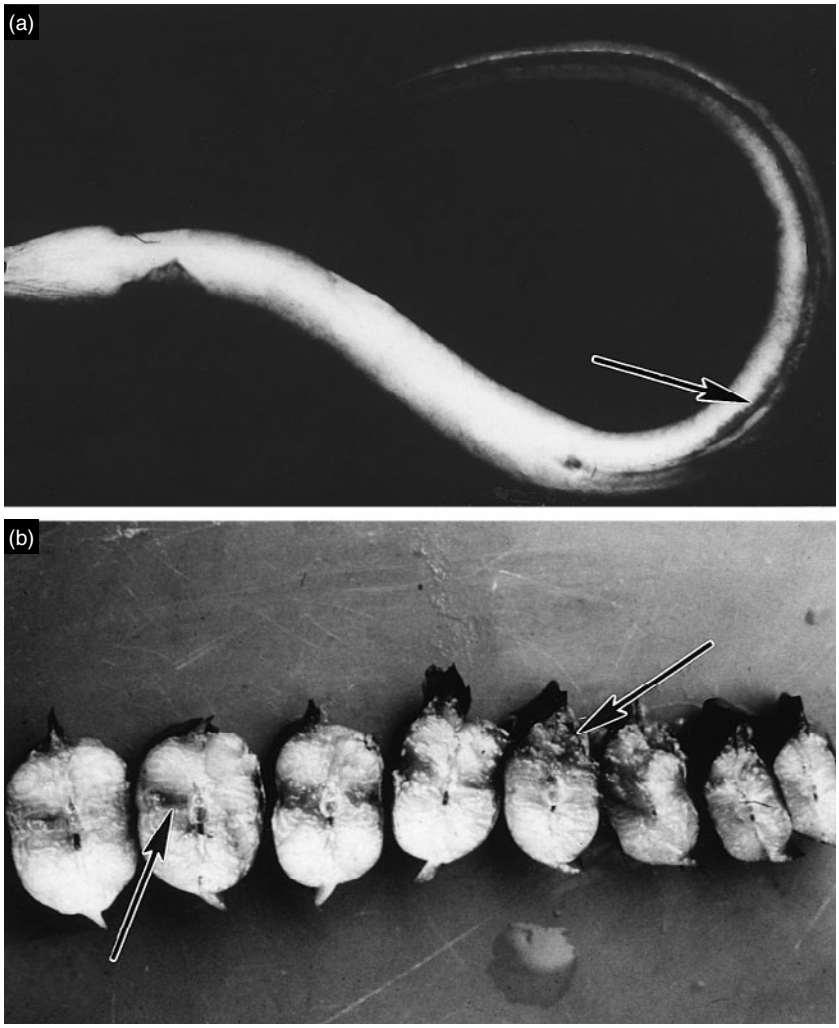


Fig. 14.2. (a) *Edwardsiella tarda* infection in Japanese eel with haemorrhagic and congested anal fin (arrow); (b) cross-sections of body of Japanese eel with inflamed and necrotic muscle lesions (arrows).

associated with *E. tarda*, including meningitis (Sonnenwirth and Kallus, 1968; Sachs *et al.*, 1974). Mizunoe *et al.* (2006) reported a rare case of empyema caused by *E. tarda*. Funada *et al.* (1988) found *E. tarda* septicaemia complicating an acute leukaemia patient in Japan, while Gilman *et al.* (1971) thought that the organism was involved with jungle diarrhoea and possibly associated with *Entamoeba histolytica* (protozoan) infection in Thailand. Van Damme and Vandepitte (1980) reported that sporadic cases of tropical diarrhoea in humans with *E. tarda* were traced to consumption of freshwater fish in Zaire. Serious and/or life-threatening infections of *E. tarda* in the muscle, which resulted from wounds received while fishing or puncture wounds caused by catfish spines, have been described in humans (Clarridge *et al.*, 1980; Hargreaves and Lucey, 1990). However, Wyatt *et al.* (1979) could not correlate *E. tarda* in aquatic

animals to human infections, but there is sufficient evidence to indicate that the organism can be a public health problem, as well as a threat to other animals such as pigs and cattle (Ewing *et al.*, 1965; Arambulo *et al.*, 1967). According to Nucci *et al.* (2002), *E. tarda* isolates from fish are similar to those of human origin; therefore, isolates from fish are potential human pathogens. Because *E. tarda* has such a wide host susceptibility and environmental adaptation, it may enter the human food chain through fish processing plants and/or poor sanitation.

Culture, immunodiagnostics and molecular techniques

E. tarda can be isolated on brain–heart infusion (BHI) agar or tryptic soy agar (TSA) with inocula from internal organs or lesions in the muscle of clinically infected fish. When incubated at 26–30°C, small, round, convex transparent colonies of approximately 0.5 mm in diameter are visible in 24–48 h. Amandi *et al.* (1982) improved isolation (from 2 to 19%) from brain of Chinook salmon by first inoculating thioglycolate media and, after incubation, transferring an inoculum to BHI agar. *E. tarda* forms small green colonies with black centres on *Edwardsiella* isolation media (EIM) (Table 14.1) and Rimler–Shotts agar medium (Shotts and Waltman, 1990; Acharya *et al.*, 2007). In addition to conventional isolation and identification with biophysical and biochemical characteristics, serological assays (fluorescent antibody technique [FAT] or enzyme-linked immunosorbent assay [ELISA]) may be satisfactory for diagnosis and identification (Meyer and Bullock, 1973; Amandi *et al.*, 1982; Swain and Nayak, 2003). Positive identification can be made using specific serum agglutination or FAT. There is no evidence of serological cross-reactivity between *E. tarda* and *E. ictaluri* (Rogers, 1981; Klesius *et al.*, 1991). Toranzo *et al.* (1987) used specific slide agglutination tests for detecting and identifying several different bacterial pathogens including *E. tarda*. Rabbit polyclonal antibodies against outer membrane proteins (OMPs) were produced and found useful in the detection of *E. tarda* by ELISA (Kumar *et al.*, 2007). These polyclonal antibodies were specific for *E. tarda* and did not cross-react with other Gram-negative bacteria. A loop-mediated isothermal amplification (LAMP) for rapid and sensitive detection of *E. tarda* was developed by Savan *et al.* (2004). This method detected *E. tarda* in the kidney and spleen of infected Japanese flounder. Seawater samples from infected flounder culture ponds were also LAMP positive.

Analyses of 16S–23S intergenic spacer regions of the rRNA operons of *E. tarda* and *E. ictaluri* revealed that a close genetic relationship between *E. tarda* and *E. ictaluri* existed and that *E. tarda* was more genetically diverse than *E. ictaluri* (Panangala *et al.*, 2005). Immunoblot analysis with goat antiserum against *E. tarda* and *E. ictaluri* epitopes also showed that *E. tarda* was more phenotypically diverse than *E. ictaluri* isolates. In another study, Panangala *et al.* (2006b) reported that there was some evidence of phenotypic diversity among *E. tarda* isolates. This report found that nine *E. tarda* isolates had 30% similarity in their protein profiles and 95% similarity in their fatty acid methyl ester profiles. Maiti *et al.* (2009), using biochemical analysis, plasmid profiling, SDS-PAGE and enterobacterial repetitive intergenic consensus (ERIC)-PCR of *E. tarda* isolated from pond sediments showed that ERIC-PCR revealed five genogroups with a similarity range from 50 to 100%, although no correlation was found between these tests. The protein patterns of *E. tarda* isolates analysed by SDS-PAGE indicated that the isolates could be divided into four groups at similarity of 40%, providing additional evidence that *E. tarda* isolates were comprised of heterogeneous groups. PCR-RFLP genotyping of 16S rDNA from *E. tarda* isolates obtained from freshwater systems demonstrated that PCR-RFLP was specific for detecting habitat isolates of *E. tarda* (Acharya *et al.*, 2007). All fish isolates were found to belong to particular genotypes, and these genotypes were not found for isolates from water or sediment. This study indicated the prevalence of site-specific genotypes in freshwater systems. In another study of 51 *E. tarda* isolates

obtained from freshwater culture systems, BOX-PCR and PCR ribotyping revealed that some of the genotypes were shown to be habitat specific (Maiti *et al.*, 2008). In conclusion, a variety of new or improved diagnostic methods are becoming available for rapid and sensitive detection of *E. tarda* in fish and culture environments.

Genome structure and transcription

The genome of *E. tarda* has not been completely sequenced. Mohanty and Sahoo (2007) have summarized some of the identified and characterized genes. The occurrence of genes for enterotoxins (*LT-I*, *EAST 1*), Shiga toxin (*Stx-1*, *Stx-2*), cytotoxic necrotizing factors (*CNF-1*, *CNF-2*), aerobactin, invasion plasmid of entero-invasive *E. coli*, EPEC adherence factor (*EAF*), intimin (*Eac*), enterohaemolysin (*EntHly*) and haemolysin (*Hly*) were analysed by PCR in 50 human and fish isolates of *E. tarda* (Nucci *et al.*, 2002). All of the isolates failed to express any of these genes. To the best of our knowledge, the occurrence of genes encoding for virulence factors has not been investigated with *E. tarda*.

A multiplex-PCR was used to detect the occurrence of tetracycline-resistance (Tc-resistance) determinants in *E. tarda* (Jun *et al.*, 2004). Among the four types of antibiotic genes found in *E. tarda*, two types, *A* and *D*, were always present on mobile plasmids and the other two genes, *B* and *G*, were located on the genomic DNA. The two Tc-resistance determinants were *A* and *D*, occurring in 55 and 45% of total antibiotic resistance *E. tarda* isolates, respectively.

Pathogenesis and immunity

Disease progression and pathology

Most pathogenesis and pathology studies of *E. tarda* have been in Japanese eel. This is in contrast to limited histopathological information in channel catfish; however, there are some reports describing *E. tarda* infections in other fish. Egusa (1976) described *E. tarda* infections of eel that spread from lesions in visceral organs into the musculature and then to the dermis. Miyazaki and Egusa (1976a,b) described histopathology of the suppurative interstitial nephritis forms of ES in adult eels in which the haematopoietic tissue of the kidney had masses of neutrophils, which contained phagocytized bacteria. Small abscesses, which developed from primary foci of neutrophils in haematopoietic tissue and nephrons, were present in the early stages of infection. Enlarged abscesses became liquefied. Bacteria spread from these abscesses to surrounding tissues, where blood vessels formed emboli that produced additional abscesses. Peripheral abscesses progressed into ulcers in the epidermis. Generalized infections showed ulcerative and necrotic (serous–exudative and liquefactive) lesions in the spleen, liver, epicardium, stomach, gill and musculature (Fig. 14.3). In the hepatitis form, micro-abscesses that contain bacteria-laden macrophages develop in the liver (Fig. 14.3). As the disease progressed, abscesses enlarged and hepatic cells became necrotic. This necrosis was followed by extensive liquefaction of abscesses and bacterial multiplication in blood vessels in various parts of the liver. Hepatic cells had fatty degeneration, while ulcers developed in the body musculature adjacent to the diseased liver (Miyazaki and Egusa, 1976b).

Histopathology of *E. tarda* in Japanese flounders, red sea bream, Japanese eels and tilapia was compared (Miyazaki and Kaige, 1985). The major difference from infection in eels was the predominance of granulomatous inflammation in the Japanese flounder and red sea bream. In infected tilapia, the abscesses in internal organs also progress to granulomas (Kubota *et al.*, 1981). Histopathology of age-0 hatchery-raised striped bass included epithelial hyperplasia, necrosis associated with the lateral line canals and abscess formation in the anterior kidney and other internal organs (Herman and Bullock, 1986). Histology of wild striped bass included ulcerative dermatitis, cardiac endothelial hyperplasia, necrotic foci

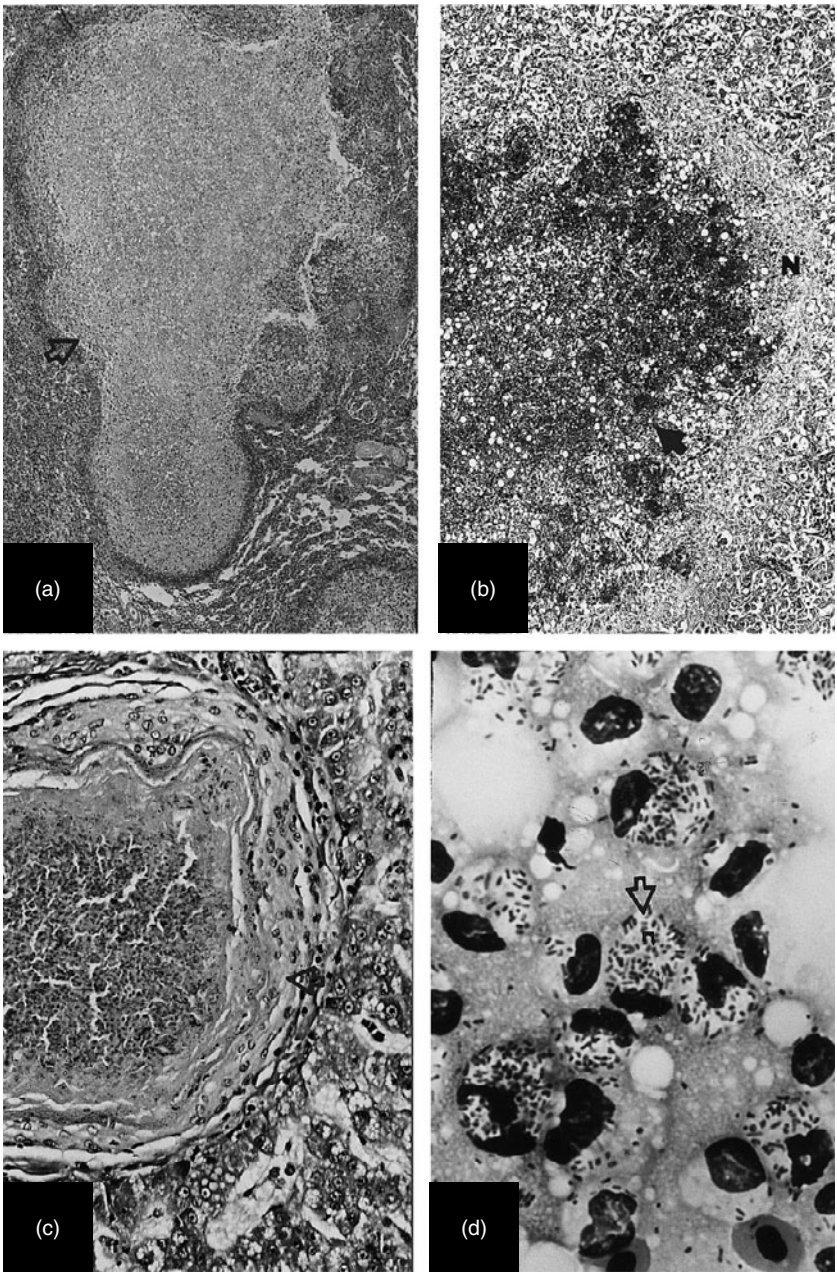


Fig. 14.3. Histopathology of *Edwardsiella tarda* infection in several different fish. (a) Abscess (arrow) in kidney of Japanese eel (haematoxylin and eosin (H & E), ×31); (b) early *E. tarda* infection in liver of tilapia. Affected hepatic cells are necrotized (N), which is followed by macrophage infiltration (arrow) (Giemsa, ×80); (c) granuloma (arrow) formation in liver of *E. tarda*-infected red sea bream (Giemsa, ×200); (d) neutrophils gorged with *E. tarda* (arrow) from abscess in the kidney of eel (Giemsa, ×1000) (all photographs by T. Miyazaki).

and granulomas in multiple organs (Baya *et al*., 1997). Padrós *et al*. (2006) characterized histopathological lesions of *E. tarda* infection in turbot as a severe inflammatory reaction with a large number of abscesses primarily in the kidney and spleen. Ultrastructural studies indicated that macrophages were the main inflammatory cell. As a result of experimental infection by immersion in *E. tarda* after mechanical skin injury, channel catfish developed a generalized congestion of internal organs and severe multifocal necrotizing inflammation of the liver, head and trunk kidney and spleen (Darwish *et al*., 2000).

Disease mechanism and virulence factors

At least some *E. tarda* isolates produce toxic extracellular products (ECP). Ullah and Arai (1983) isolated an exotoxin from *E. tarda* culture media and found no evidence of endotoxins, therefore postulating that the exotoxin was responsible for pathogenicity. However, factors that regulate pathogenicity of *E. tarda* are unclear. Suprapto *et al*. (1995, 1996) detected a heat-labile ECP in a virulent strain of *E. tarda* belonging to serogroup A, which was lethal to Japanese eel and Japanese flounder. The optimum incubation temperature for ECP production was 25–30°C, which coincided with most reports of optimum temperature for fish susceptibility. An intracellular component (ICC) was also detected in bacteria and was associated with cell lysis. Japanese flounder appeared to be more susceptible to *E. tarda* (about 15 times higher) than the Japanese eel. Results of these studies suggest that the toxin produced by *E. tarda* plays an important role in its virulence. Han *et al.* (2006) sequenced the superoxide dismutase gene (*sodB*) and catalase gene (*katB*) and reported that virulent *E. tarda* isolates possessed type I *sodB* and *katB*, whereas non-virulent isolates had type II *sodB* but not *katB*. No difference in haemolytic and cytotoxic activities between virulent and non-virulent isolates was observed. The LD_{50} values of virulent *E. tarda* isolates were reported to range between $10^{2.5}$ and $10^{5.5}$ cfu/olive flounder. They further demonstrated the ability of *E. tarda* to resist complement activity and that phagocytosis was conferred by superoxide dismutase and catalase activities, indicating that these two enzymes played an essential role in the pathogenicity of *E. tarda*.

The ability of *E. tarda* to infect warm-blooded animals, humans in particular, was shown by Janda *et al*. (1991), who demonstrated its ability to invade HEp-2 cell monolayers, produce cell-associated haemolysin and siderophores and express mannose-resistant haemagglutination against guinea-pig erythrocytes. Some strains of *E. tarda* were virulent to mice. Janda and Abbott (1993a) showed that strains of *E. tarda*, a known pathogen of warm-blooded animals, produced 30–40% higher levels of cell-associated haemolytic activity (haemolysins) than strains of *E. ictaluri*. The increased haemolytic activity could contribute to the pathogenicity of *E. tarda* to humans. When grown under iron-restricted conditions in the presence of ethylenediamine di (*o*-hydroxyphenylacetic acid), haemoglobin, haematin and haemin stimulated bacterial growth in both liquid and agar bioassays. Haemolysin activity under these conditions was increased three- to >40-fold. All of these studies indicate that, in addition to its invasive capabilities, *E. tarda* produces a haemolysin, which is partially regulated by the availability of iron and may also play a role in human disease (Igarashi *et al*., 2002).

Immunity

Enhanced innate immunity against *E. tarda* has been produced by the administration of immunostimulants and probiotic bacteria in fish. Park and Jeong (1996) evaluated a protein-bound polysaccharide preparation (PS-K) in tilapia to produce innate immunity against *E. tarda*. The PS-K was isolated from a mushroom, *Coriolus versicolor*, and given to tilapia by i.p. injection. The results indicated that PS-K activated non-specific immunity and protection against *E. tarda*. Similar results were demonstrated in blue tilapia (*Tilapia aureus*) and grass carp (*Ctenopharyngodon idella*) to *E. tarda* after administration of polysaccharides (Wang

and Wang, 1997). The efficacy of dietary β-glucan on innate immunity against *E. tarda* was evaluated by Misra *et al.* (2006). They found that 250 mg of β-glucan/kg diet enhanced innate immunity, growth and survival of Indian major carp fingerlings. The probiotic bacterium, *Lactobacillus rhamnosus*, enhanced an alternative complement system, phagocytosis and protected tilapia against *E. tarda* (Pirarat *et al.*, 2006).

Fimbrial protein (FP), OMPs and lipopolysaccharide (LPS) are recognized as important antigens of *E. tarda*. Sakai *et al.* (2003) investigated the haemagglutinating properties of *E. tarda* isolated from fish. They reported fimbriae were involved in the haemagglutination of *E. tarda*. Immunoblot analyses of 18 isolates of *E. tarda* revealed that a 19.4 kDa protein was responsible for haemagglutinating activity, and this protein was identical to the fimbrial subunit. Virulent *E. tarda* may use this fimbrial protein to adhere to the body and gill mucus layers during the infectious process. Thus, immune response to this protein may inhibit infection. Aoki and Holland (1985) reported that OMPs of *E. tarda* were similar in their protein profiles. The dominant OMPs had molecular sizes of 36 and 46 kDa and, when cultured under iron-limiting conditions, three OMPs of 68, 73 and 84 kDa were found. Another important immunodominant antigen is LPS. The LPS of a virulent isolate of *E. tarda* was investigated to determine the structural characteristics of its *O*-polysaccharide antigen (Vinogradov *et al.*, 2005). The LPS was found to be a polymer of repeating pentasaccharide units. This information will be useful for the production of an LPS subcomponent or modified LPS live vaccine.

Macrophages are believed to play a role in immunity against Gram-negative bacteria. However, Ishibe *et al.* (2008) found that virulent *E. tarda* prevented the activation of the reactive oxygen species (ROS) generation system during phagocytosis and reduced the chemiluminescence response. Also, the virulent *E. tarda* was more resistant to H_2O_2. Overall, these results indicate that virulent *E. tarda* have the ability to survive and multiply within macrophages and this leads to dissemination of *E. tarda* throughout the host during infection. When immune gene expressions in leucocytes from Japanese flounder were investigated by Matsuyama *et al.* (2007), ten genes were found to be upregulated in immunized flounder and 11 genes were upregulated in non-immunized fish. They found no evidence to link different gene expression patterns and the efficacy of vaccination.

Exposure of macrophages to ECP from *E. tarda* was found to promote chemotactic activities (Wiedenmayer *et al.*, 2008). This activity may be involved in the inflammatory responses of tilapia to *E. tarda*. A recent study revealed a relationship between lymphoid apoptosis and the inflammatory response during *E. tarda* infection (Pirarat *et al.*, 2007), showing that *E. tarda* caused systemic immunosuppression of lymphoid tissues which resulted in a suppression in the inflammatory response during the initial step of septicaemia. Additional studies are needed to determine the role of antibody and cellular immunities in *E. tarda* infection.

Epidemiology, treatment and control

Epidemiology

The source of *E. tarda* is presumably the intestinal contents of carrier animals, but it may be a common inhabitant of the aquatic environment. In the USA, *E. tarda* was isolated from 75% of water samples, 64% of pond-mud samples and 100% of frogs, turtles and crayfish from catfish ponds (Wyatt *et al.*, 1979). Following processing, *E. tarda* was also isolated from up to 88% of cultured channel catfish fillets and from 30% of imported fish fillets in the USA. In an ecological study of *E. tarda* in a salt-water flounder farm, Rashid *et al.* (1994a) isolated the bacterium from 86% of water samples from one pond and 22% of water samples from a second pond. The organism was present in 44% of sediment samples and 14% of fish from the first pond, and 0% of sediment samples and 2% of fish from the second pond. Clinical ES did not occur in

the fish in either pond. The presence of *E. tarda* in fish may be associated with its presence in the environment, or the high incidence in the environment may have been a function of the organism's presence in the fish. Hidaka *et al.* (1983) showed that *E. tarda* cell counts in eel-pond water at 22–26°C were four times higher when clinical disease was present than when there was no disease.

In spite of the apparent extensive presence of *E. tarda* in fertile channel catfish ponds, infection in these fish is uncommon. When deaths do occur, mortality seldom exceeds 5%; however, if the fish are moved into confined holding tanks, the proportion of infection may increase quickly to 50%, with concomitant deaths (Meyer and Bullock, 1973). Mortality data of *E. tarda* infected cultured eels in Asia indicate potentially high losses. Ishihara and Kusuda (1981) induced up to 60% cumulative mortality in 100 g eels infected experimentally by immersion in water containing *E. tarda*. In a naturally infected Japanese eel population, the percent cumulative mortality was 80 (Kodoma *et al.*, 1987). Percent cumulative mortality of Nile tilapia infected spontaneously in confinement reached 83 in 4 days (Benli and Yildiz, 2004).

Edwardsiella septicaemia of catfish in the USA occurs mostly during the warm summer months. Although Egusa (1976) reported that *E. tarda* infections of eels in Japan were also more prevalent during the summer, Liu and Tsai (1980) found that infections of eels in Taiwan were most common when water temperatures were 10–18°C, during January–April. Although environmental stressors are not essential precursors to *E. tarda* infections in fish, high temperature, poor water quality and high organic fertility probably contribute to the onset and severity of the disease. Juvenile channel catfish that were infected experimentally with *A. hydrophila* and then exposed to environmental stressors (low dissolved oxygen, high ammonia and high carbon dioxide) developed *E. tarda* infections in 25–50% of the fish, compared with 4.5–12.5% *E. tarda* infections in the non-*A. hydrophila*-injected or otherwise non-stressed fish (Walters and Plumb, 1980). Indications were that environmentally induced stress and other bacterial infections could predispose channel catfish to endemic *E. tarda*. Many epidemics associated with *E. tarda* infection occur in fish exposed to fluctuating water temperatures (Liu and Tsai, 1980; Amandi *et al.*, 1982) or fish in highly enriched waters (Meyer and Bullock, 1973; Baya *et al.*, 1997; Uhland *et al.*, 2000). Cultured tilapia exposed to stressors are also susceptible to *E. tarda* (Benli and Yildiz, 2004). On several occasions, intensively cultured and environmentally stressed Nile tilapia in recirculating systems that had a moderate to heavy *Trichodina* (protozoan parasite) infestation were also infected with *E. tarda* and *Streptococcus* spp. (J.A. Plumb, unpublished). While stressed, neither bacterial infection responded to chemotherapy, but as soon as the stressor was relieved and parasites were eliminated, the *E. tarda* infection disappeared.

Transmission control

The first step in controlling most infectious diseases in the aquatic environment is through health management, which includes avoiding contact between pathogen and host, management of the environment by reducing stressful conditions and removing sick and dead fish as soon as possible. *E. tarda* infections are no exception. Also, utilization of prophylactic treatments and implementation of sanitary aquaculture practices, judicious use of legal drugs and chemicals when infections occur, application of available vaccines and use of genetically improved stocks are aids in health management (Plumb, 1999b). Because *E. tarda* is a non-obligate pathogen, it is not possible in most instances to eliminate completely or prevent the organism's presence totally. For example, preventing infected animals (e.g. undesirable fish, turtles, snakes) from coming into contact with the aquaculture species is impractical, except under certain circumstances, such as closed recirculating systems. Maintaining a suitable oxygen concentration and low carbon dioxide and ammonia, reducing water

enrichment, preventing wide temperature fluctuations and providing a proper diet are basic to health management. Although these goals are difficult to attain in intensive or commercial aquaculture, they should be pursued. Incongruently, with regard to *E. tarda*, while environmental stress is undesirable, Caruso *et al.* (2002) showed that stress-induced increases in plasma lysozyme in sheatfish (*Silurus glanis*) did not increase susceptibility to infection. Plumb (1984) also suggested a low pathogenic action of this organism under stressful conditions.

Chemotherapy

Therapy of ES is by oral application of drugs in feeds of cultured fish. Two drugs, oxytetracycline and a potentiated sulfonamide (sulfadimethoxine–ormetoprim in a 5:1 ratio), are approved by the US Food and Drug Administration (FDA) for some bacterial diseases of some fish; however, neither of these drugs is approved specifically for *E. tarda* infections in any species of fish (Schnick *et al.*, 1989). Oxytetracycline (Terramycin) is fed at 50 mg of drug/kg of fish/day for 12–14 days, followed by a 21-day withdrawal period before fish are processed for human consumption. Sulfadimethoxine–ormetoprim (Romet-30) is also used as a feed additive to deliver 50–100 mg of drug/kg/day for 5 days, requiring only 3 days withdrawal for catfish that are skinned prior to being marketed, but up to 42 days withdrawal is required if fish are not skinned during processing. Because of a possible lack of palatability of feed with the higher dose of sulfadimethoxine–ormetoprim, it was suggested by Johnson and Smith (1994) that the amount of drug be reduced and feed level increased. Both of these drugs are generally effective against *E. tarda* infections as long as treatment is initiated prior to the disease advancing to a point when fish stop feeding. Some countries have a wider range of antibiotics available with which to treat bacterial infections. Resistance of bacteria to antibiotics is a constant problem in cultured fish. Fifty-four strains of *E. tarda* from cultured eels in Taiwan were tested *in vitro* for their susceptibility to a large number of antibiotics (Chen *et al.*, 1984). They found a high rate of susceptibility of *E. tarda* to gentamicin sulfate and nalidixic acid and a high rate of resistance to erythromycin and all sulfa drugs. Waltman and Shotts (1986a) tested 116 isolates of *E. tarda* from the USA and Taiwan for their susceptibility to 37 antimicrobials and found a higher number of resistant isolates from Taiwan. Antibiotic sensitivity testing of only five *E. tarda* isolates from catfish and tilapia in the USA by Plumb *et al.* (1995) showed that all were sensitive to sulfadimethoxine–ormetoprim and four were sensitive to oxytetracycline.

Antibiotic resistance of some *E. tarda* isolates from Taiwan was plasmid mediated (Aoki *et al.*, 1977; Aoki and Kitao, 1981). Aoki *et al.* (1986) detected 13 transferable R plasmids in *E. tarda*, two of which encoded for resistance to chloramphenicol, tetracycline and sulfonamide. Subsequent studies showed that 20% of 152 antibiotic-resistant strains of *E. tarda* possessed transferable antibiotic-resistant R plasmids; therefore, the function of some plasmids in the bacterium was unknown (Aoki *et al.*, 1987). When R plasmid resistance to tetracycline and sulfonamides occurred, Aoki *et al.* (1989) recommended that a potentiated sulfonamide at 25 mg/kg/day, oxalinic acid at 12.5 mg/kg/day or miloxacin at 6.2 mg/kg/day be fed. Liu and Wang (1986) reported that nearly 92% of *E. tarda* isolates from the water of eel-culture ponds showed some degree of antibiotic resistance. *E. tarda* has a long history of causing disease in Southeast Asian aquaculture, where the pathogen has had greater exposure to a variety of antibiotics; therefore, higher resistance may be encountered (Aoki *et al.*, 1989).

Vaccination

Immunization has become a popular method for controlling and preventing numerous diseases of fish (Ellis, 1988; Anderson, 1992; Mekuchi *et al.*, 1995b; Press and Lillehaug, 1995) and Newman (1993) predicted that vaccines for a variety of fish diseases would become available. Salati (1988) reviewed the techniques and procedures for vaccinating

eels against *E. tarda* and stated that the two basic types of vaccines were whole-cell bacterins and bacterial extracts. Immunization of eels against *E. tarda* was first investigated by Song and Kou (1981). Song *et al.* (1982) reported that a single immersion of 6 g elvers was effective, but that two or three exposures to the vaccine were more effective in eliciting immunity and protection, which lasted 10 weeks. Salati *et al.* (1983) immunized eels with LPS, culture filtrates and formalin-killed whole cells (FKC). They concluded that LPS was the immunogenic component, but it was later shown that polysaccharide without the lipid component was more antigenic than other preparations (Salati and Kusuda, 1985a,b). In another study, immunization of eels by injection of FKC and LPS from *E. tarda* showed only slight to moderate protection from challenge by injection with virulent *E. tarda* 21 days after vaccination (Gutierrez and Miyazaki, 1994). Kawahara *et al.* (1990) concluded that LPS was the more effective immunogen and Newman (1993) suggested that the serologic heterogeneity of *E. tarda* was likely to limit the extent of strain protection of both FKC and LPS vaccines.

Rashid *et al.* (1994b) vaccinated Japanese flounder with formalin-killed *E. tarda* and extracellular and intracellular components by i.m. injection, bath immersion and oral exposure. While the extracellular and intracellular components were lethal to the flounder, the serum agglutinating antibody titres against FKC rose in all other immunized groups, except those vaccinated by immersion. Protection was not demonstrated clearly with the FKC, but death of challenged fish was delayed in the fish immunized by immersion and injection. Fry and fingerlings of carp (*L. rohita* and *C. catla*) bath immersion immunized with FKC of *E. tarda* were shown to be protected against infection (Swain *et al.*, 2002) and a serum antibody response in Indian major carp was measured (Swain *et al.*, 2007). The antibody was detected at 14 days post-immunization (PI) and rose to highest levels at 4 weeks PI before declining after 8 weeks PI. The immunized fish were challenged with live *E. tarda* at 8 weeks and the relative percent survival (RPS) was 80. These results suggest that FKC may provide protection. Salati *et al.* (1987) concluded that immunization with LPS and FKC by i.m. injection enhanced the phaogocytic response of sea bream and that this response was one of the most important defence mechanisms against *E. tarda*. However, Mekuchi *et al.* (1995b) concluded that LPS and FKC vaccines administrated by injection did not produce acceptable protection. Salati (1988) stated that 'bacterins which are simple and cheap to produce on a large scale do provide protection against anquillettes and while the protection is not complete, it may prove useful enough to farms with severe *Edwardsiella* problem'. The major limitations of *Edwardsiella* bacterins are incomplete protection, variable efficacy, short duration of protection and lack of broad strain protection. The lack of protection may be overcome by a 37 kDa OMP, which produces effective protection against different serotypes of *E. tarda* (Kawai *et al.*, 2004). Japanese flounder immunized with injection of OMP had a significantly higher survival rate than the non-immunized fish. A specific antibody titre against this OMP was measured in the immunized fish. Previously, Tu and Kawai (1999) identified OMP as one of the protective antigens of *E. tarda*. It appears that *E. tarda* has several protective antigens that may include but are not limited to OMP, LPS and extracellular toxins or substances.

Other approaches to design an effective vaccine against *E. tarda* include a vaccine containing adjuvant and an aqueous bacterin (Castro *et al.*, 2008). The results of this study showed that the non-mineral oil adjuvanted vaccine given by i.p. injection conferred RPS of greater than 90 against *E. tarda* infection in cultured turbot. Efficacy of oral immunization of olive flounder with *E. tarda* ghosts (ETG), produced by lysis of live cells with a lysis plasmid, was tested by Kwon *et al.* (2007). The ETG immunized olive flounder had a specific antibody response and high survival to challenge infection. The authors concluded that ETG immunization induced a protective adaptive immunity.

A live *E. tarda* vaccine was developed by Igarashi and Iida (2002) and assessed for its ability to protect tilapia against ES. A suicide delivery plasmid with a transposon in its plasmid DNA was used to produce a low producing siderophore mutant. This mutant was not completely avirulent but, following i.p. vaccination, 5 out of 50 fish died following challenge with virulent *E. tarda*. Specific antibody response was stimulated following injection of these vaccines, suggesting that cell-mediated immunity was activated following immunization and might be required for successful vaccination against *E. tarda*. The importance of cell-mediated immunity in protection against *Edwardsiella* following vaccination with modified live *E. ictaluri* vaccine has been demonstrated previously by Klesius and Shoemaker (1999). Lan *et al.* (2007) constructed a live attenuated esrB mutant of *E. tarda* and demonstrated its potential as a vaccine against haemorrhagic septicaemia in turbot. They found that RPS was greater than 50 and antibody titres correlated with protection. Igarashi and Iida (2002) concluded that less virulent and safer strains of *E. tarda* for live vaccines should be produced in the future. An attenuated live *E. tarda* vaccine that can stimulate long-term protection following bath immersion administration may be used ultimately to prevent ES. More recently, a modified live vaccine against *E. tarda* has been developed and tested in tilapia (Evans *et al.*, 2006). This modified live vaccine was given by bath immersion and provided protection to tilapia. In conclusion, a vaccine against *E. tarda* has undergone years of research and development, but a commercial vaccine is not available.

Enteric Septicaemia of Catfish (*Edwardsiella ictaluri*)

Enteric septicaemia of catfish (ESC), caused by the bacterium *E. ictaluri*, was reported by Hawke (1979), in channel catfish from the USA (Hawke *et al.*, 1981); however, the bacterium may have been present in cultured catfish as early as the late 1960s (Mitchell and Goodwin, 1999). Since its discovery, this disease has become one of the most important infectious diseases of the commercial catfish industry in the USA, especially in the south-east (Wagner *et al.*, 2002). Estimates of the cost of the disease to the catfish industry have ranged from US$20–60 million annually (Plumb and Vinitnantharat, 1993; Shoemaker *et al.*, 2003), but a carefully calculated assessment of losses is not available. Because of its comparatively narrow host specificity, ESC is not a great economic problem in regions where channel catfish are not cultured, although the bacterium has been isolated from other catfish species and species of fish in the wild and other parts of the world (Plumb, 1999a,b) (Table 14.4). *E. ictaluri* should not be confused with *E. tarda*, another member of the same genus that is frequently found in aquatic animals and is responsible for opportunistic infections in fish and mammals, including humans.

The disease agent

Current classification

E. ictaluri (type strain ATCC 33202), described by Hawke *et al.* (1981), is a typical member of the Enterobacteriaceae in most respects; it is a Gram-negative, short, pleomorphic rod, which measures about 0.75×1.5–2.5 μm. It is weakly motile by means of peritrichous flagella at 25–30°C, but not at higher temperatures. The organism is catalase-positive, cytochrome oxidase-negative, ferments glucose and reduces nitrate to nitrite (Shotts and Teska, 1989). It is also lactose- and indole-negative and produces an alkaline slant and acid butt without H_2S on TSI agar. Although *E. ictaluri* ferments and oxidizes glucose while producing gas at 20–30°C but not at 37°C, the organism is non-reactive on most sugars and is intolerant of NaCl higher than 1.5% in culture medium (Table 14.1). Growth on culture media requires 36–48 h to form punctate colonies on BHI agar at 28–30°C; it grows poorly, if at all, at 37°C. *E. ictaluri* and *E. tarda* may be differentiated

from each other biochemically by the production of indole and hydrogen sulfide (*E. ictaluri* does not produce these, while *E. tarda* does). Furthermore, *E. ictaluri* does not grow well at 37°C, whereas other members of the Enterobacteriaceae, *Yersinia ruckeri*, *Hafnia alvia*, *E. tarda* and *E. hoshinae*, grow well at 37°C. For the most part, *E. ictaluri* is aerobic, but Mitchell and Goodwin (2000) have described multiple isolates that possess anaerobic metabolism.

Biology

When first described, *E. ictaluri* was thought to be an obligate pathogen, unable to live for an extended period outside the host (Hawke, 1979). However, later work indicated that the organism could survive in sterilized pond-bottom mud for over 90 days at 25°C (Plumb and Quinlan, 1986) (Fig. 14.4). The survival of *E. ictaluri* in the environment may be influenced by microbial competition, because Earlix (1995) showed that the organism did not survive well in water or bottom soils containing other microbes. In spite of these data, *E. ictaluri* is still generally considered an obligate pathogen (Plumb, 1999a).

Phenotypic, antigenic and serological characteristics

By most accounts, *E. ictaluri* appears to be a rather homogeneous species biophysically, biochemically, genetically and perhaps serologically (Waltman *et al.*, 1986b; Newton *et al.*, 1988; Starliper *et al.*, 1988; Bertolini *et al.*, 1990; Panangala *et al.*, 2005). Waltman *et al.* (1986b) and Plumb and Vinitnantharat (1989) found almost no differences in biophysical or biochemical characteristics among many isolates of *E. ictaluri* from a variety of species of fish and geographical regions. Data presented by Rogers (1981), Plumb and Klesius (1988), Bertolini *et al.* (1990) and Vinitnantharat and Plumb (1993) showed little serological diversity using antisera generated in mice, goat or rabbits. Chen and Light (1994) reported no cross-reactivity of *E. ictaluri*-specific antibodies in domesticated or wild channel catfish to nine other fish bacterial pathogens, nor did fish immunized with these nine pathogens possess antibody titres to *E. ictaluri*.

Lobb and Rhoades (1987) found that *E. ictaluri* possessed at least two DNA plasmids and reported some serological and

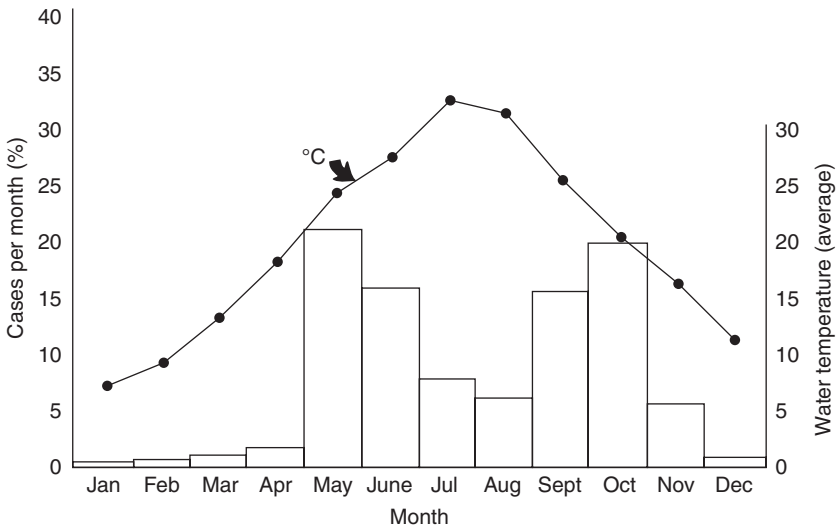


Fig. 14.4. Survival of *Edwardsiella ictaluri* in pond water and mud at different temperatures (from Plumb and Quinlan, 1986; reprinted with permission of the American Fisheries Society).

possibly plasmid differences in *E. ictaluri* strains. Subsequently, Lobb *et al.* (1993) utilized plasmid and serological methods to show that differences existed between *E. ictaluri* strains, particularly those isolated from non-ictalurid fishes. Newton *et al.* (1988) found five *E. ictaluri* isolates that contained one to three plasmids each, based on their molecular mass. It was proposed by Reid and Boyle (1989) that all *E. ictaluri* isolates had plasmids. The role of all plasmids in *E. ictaluri* is not known; however, Starliper *et al.* (1993) have transferred antibiotic resistance from *Escherichia coli* to *E. ictaluri* via plasmids by cohabitation of the two bacteria. The transfer frequency was 1.197×10^{-2} transconjugates/donor cell in the mating mixture. In a companion study, Cooper *et al.* (1993) demonstrated that the plasmids of *E. coli* and *E. ictaluri* were either identical or very similar. Contrastingly, plasmids of *E. ictaluri* were thought to be specific enough for Speyerer and Boyle (1987) to suggest that they could be used as a deoxyribonucleic acid (DNA) probe to detect *E. ictaluri* in fish. This may be possible because plasmids pEI1 and pEI2 have been fully sequenced (Fernandez *et al.*, 2001).

Klesius and Shoemaker (1997) suggested antigenic differences based on challenge experiments conducted in channel catfish. Immunization with live *E. ictaluri* isolates did not result in protection against heterologous isolates in some cases. The use of monoclonal antibody and colloidal gold to localize the predominant antigens of *E. ictaluri* showed that the organism possessed major outer membrane antigens, with molecular masses of 60 and 36 kDa (Plumb and Klesius, 1988; Newton *et al.*, 1990; Klesius and Horst, 1991). *E. ictaluri* has peritrichous flagella and pili have been detected occasionally in scanning electron micrographs. Newton and Triche (1993) isolated and purified two proteins from the flagella of *E. ictaluri*, with apparent molecular mass of 42 and 38 kDa, respectively. These authors also showed that the LPS from 40 different *E. ictaluri* isolates were all the same when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and an immunoblot analysis revealed a high degree of antigenic similarity among the isolates. However, according to Saeed and Plumb (1987), the LPS of *E. ictaluri* was different from that of other Enterobacteriaceae, and Weete *et al.* (1988) demonstrated a rough LPS that had no 'O' side-chains.

A phenotypic comparison using FAME and Biolog revealed a high degree of similarity between tadpole madtom (*Noturus gyrinus*) and channel catfish *E. ictaluri* isolates. However, amplified fragment length polymorphism (AFLP) analysis revealed madtom isolates had a distinctly different AFLP fingerprint from catfish isolates, which indicated a certain extent of genetic diversity within the species (Klesius *et al.*, 2003). Using an enterobacterial repetitive intergenic consensus (ERIC) PCR primer, Bader *et al.* (1998) showed that among 20 *E. ictaluri* isolates, four subgroups were identified. Genetic analysis of the 16S–23S intergenic spacer regions (ISRs) of the rRNA operons of *E. ictaluri* and *E. tarda* isolates from fish has revealed a high degree of sequence similarity of the 16S–23S ISRs of both species (Panganala *et al.*, 2005). However, they found a higher degree of sequence homogeneity among *E. ictaluri* compared with *E. tarda*.

Transmission

In aquaculture, infected channel catfish are the primary source of *E. ictaluri*, with natural transmission occurring primarily through the water. Horizontal transmission of *E. ictaluri* was demonstrated by Klesius (1994). Clinical infections occurred 12 days post-exposure to fish that had died of ESC. Experimental transmission of *E. ictaluri* is achieved by waterborne exposure, i.m. or i.p. injection, intestinal intubation or introducing the bacterium into the nares only (Plumb and Sanchez, 1983; Shotts *et al.*, 1986; Newton *et al.*, 1989; Morrison and Plumb, 1994). Acute clinical disease usually appears at 5–7 days post-bath exposure at 25°C.

The nares are a primary site of *E. ictaluri* invasion, and exposure of this organ to *E. ictaluri* can initiate chronic ESC (Morrison

and Plumb, 1994). Mgolomba and Plumb (1992) and Klesius (1992) showed that survivors of an epidemic still carried *E. ictaluri* long after clinical disease had disappeared; therefore, they could serve as reservoirs of the pathogen. Research indicated that, in a pond where fish were dying of *E. ictaluri*, the water in the vicinity of dead fish had significantly higher *E. ictaluri* counts than water where there were no carcasses (Earlix, 1995). Removal of dead fish should reduce the number of bacteria to which non-infected fish are exposed. Transmission from adults to offspring during spawning is likely but remains as yet unproven.

Once *E. ictaluri* is introduced into a particular body of water, the bacterium probably remains there as a source of infection, either in carrier fish or in the environment. The means by which *E. ictaluri* is transferred from farm to farm is speculative, but it is likely that the transfer of infected fish is the primary instrument of transmission. Fomites, for example, seines, nets and other equipment that are not disinfected or thoroughly air-dried between use could be sources of infection (Plumb, 1999a).

Fish from a population that has recovered from the disease are considered to be immune carriers that may have high levels of *E. ictaluri*-specific antibodies. Occasional losses due to recurrent ESC will occur in these populations, especially after stress is induced. *E. ictaluri* has been detected in the kidney of such fish well over 4 months after exposure (Klesius, 1992; Antonio and Hedrick, 1994), suggesting that carrier fish act as the natural reservoir for the organism. It is believed that shedding with faeces is the main mode of dissemination into the environment. Pathogen persistence and the common practice of continual partial harvest and stocking within a production pond have contributed to the success of this pathogen and the prevalence of ESC in the catfish aquaculture industry. Moreover, the agent can survive in pond sediments for an extended period of time (Plumb and Quinlan, 1986) and this may be another important factor in repeated epidemics in given areas. Researchers have also found the bacterium in the gut of fish-eating birds by performing fluorescent antibody tests on ingesta (Taylor, 1992; Waterstrat *et al.*, 1999). However, few *E. ictaluri* could be cultured, indicating that not all of the bacteria were viable. Therefore, birds may not be an important means of disseminating this pathogen.

Geographical distribution and host range

E. ictaluri infection has been confirmed only in the USA, Thailand (Kasornchandra *et al.*, 1987) and Singapore (Humphrey *et al.*, 1986). However, it may also exist in Taiwan (Chung and Kou, 1983), Vietnam (Crumlish *et al.*, 2002), China (Tan *et al.*, 2003), Spain (Blanch *et al.*, 1990) and Croatia (Popovíc *et al.*, 2001). There have also been unconfirmed reports of clinical signs typical of ESC in other parts of the world where catfish are cultured (Table 4.4). In the USA, *E. ictaluri* is found primarily across the south-eastern region, where channel catfish are grown commercially (i.e. Alabama, Arkansas, Louisiana, Mississippi, Texas, Florida, Georgia, North Carolina and South Carolina). However, the bacterium has been reported to cause disease among cultured channel catfish in other states, such as Arizona, California, Idaho, Indiana, Kansas, Maryland, New Mexico and Virginia. The bacterium was isolated recently from wild tadpole madtom (*N. gyrinus*) in New Jersey (Klesius *et al.*, 2003). With the continual worldwide dissemination of channel catfish for aquaculture purposes, it is likely that *E. ictaluri* will be reported from other geographical regions (Plumb, 1999a,b).

E. ictaluri has a narrower host range than that of *E. tarda*; however, it is still diverse. Four families of catfish: Ictaluridae (North American freshwater catfish), Clariidae (air-breathing catfish), Siluridae (shark catfish) and Pangasiidae (sheatfish) in the Order Siluriformes (catfish) have been infected either naturally or experimentally (Table 14.4). Most reported cases of disease caused by *E. ictaluri* are in channel catfish, but the bacterium has been isolated from related North American freshwater catfish including blue catfish (*I. furcatus*), white catfish

Table 14.4. Families and species of fish, habitat – aquarium (A), cultured (C), wild (W) or experimental (E) – and countries from which *Edwardsiella ictaluri* has been reported.

Fish families and species	Habitat	Country	Reference
Ictaluridae (N. American freshwater catfish)			
Blue catfish (*Ictalurus furcatus*)	E	USA	Wolters and Johnson (1994)
Brown bullhead (*Ameiurus nebulosus*)	W	USA	Hawke *et al.* (1981); Plumb and Sanchez (1983)
Channel catfish (*Ictalurus punctatus*)	C/ W	USA	Hawke (1979); Hawke *et al.* (1981); Chen *et al.* (1994)
Tadpole madtom (*Noturus gyrinus*)	W	USA	Klesius *et al.* (2003)
White catfish (*Ameiurus catus*)	W	USA	Plumb and Sanchez (1983); Hawke *et al.* (1981)
Clariidae (air-breating catfish)			
Walking catfish (*Clarias batrachus*)	W	Thailand	Kasornchandra *et al.* (1987)
Pangasiidae (shark catfish)			
Asian catfish (*Pangasianodon hypophthalmus*)	C	Vietnam	Crumlish *et al.* (2002); Tan *et al.* (2003)
Siluridae (sheatfish)			
European catfish (Wels catfish) (*Silurus glanis*)	E	USA	Plumb and Hilge (1987)
Non-catfish families			
Bengal danio (*Danio devario*)	A	USA	Blazer *et al.* (1985); Waltman *et al.* (1985)
Blue tilapia (*Oreochromis aureus*)	E	USA	Plumb and Sanchez (1983)
Chinook salmon (*Oncorhynchus tshawytscha*)	E	USA	Baxa *et al.* (1990)
European sea bass (*Dicentrarchus labrax*)	C	Spain	Blanch *et al.* (1990)
Green knife fish (*Eigemmannia virescens*)	A[a]	USA	Kent and Lyons (1982)
Japanese eel (*Anguilla japonica*)	C	Taiwan	Chung and Kou (1983)
Rosy barb (Puntius) (*Puntius conchonus*)	A[b]	Singapore	Humphrey *et al.* (1986)
Rainbow trout (*Oncorhynchus mykiss*)	E	USA	Baxa *et al.* (1990)
Rudd (*Scardinius erythrophthalmus*)	W	Croatia	Popovíc *et al.* (2001)
Striped bass (*Morone saxatilis*)	E	USA	Baxa *et al.* (1990)
White perch (*Morone americana*)	E	USA	Pasnik *et al.* (2007)

[a]Maintained in aquaria for neurophysiological studies; [b]imported to Australia.

(*Ameiurus catus*), brown bullhead (Hawke *et al.*, 1981) and wild tadpole madtom (Klesius *et al.*, 2003). *E. ictaluri* has also been reported from other families of catfish including walking catfish and Asian catfish (*Pangasianodon hypothalamus*) from Asia (Kasornchandra *et al.*, 1987; Crumlish *et al.*, 2002). Eleven species in seven non-catfish families have been reported to be infected by *E. ictaluri*, including cultured European sea bass (Blanch *et al.*, 1990) and Japanese eel (Chung and Kou, 1983), wild rudd (*Scardinius erythrophthalmus*) (Popovíc *et al.*, 2001) and several ornamental species: Bengal danio (*Danio devario*) (Blazer *et al.*, 1985; Waltman *et al.*, 1985), green knife fish (*Eigemmannia virescens*) (Kent and Lyons, 1982) and rosy barb (Humphrey *et al.*, 1986).

Cultured channel catfish are the most severely affected, but less susceptible Ictalurids include white catfish, blue catfish and brown bullhead. Although ESC generally is considered a disease of cultured catfish, its occurrence in wild tadpole madtom as well as wild channel catfish in California (Chen *et al*., 1994) and rudd in Croatia (Popovíc *et al*., 2001) suggest the pathogen can occur in other wild species of fish.

Experimental infections in blue catfish have been difficult to induce, but an occasional natural infection has occurred in this species. Variation in susceptibility in channel catfish strains has been demonstrated (Wolters and Johnson, 1994). Additionally, Wolters *et al*. (1996) showed that, following experimental infection, channel catfish had the lowest survival (62%) and blue catfish had the highest survival (90%), while survival of hybrids of the two species was intermediate (74%). Plumb and Hilge (1987) demonstrated that the European catfish (sheatfish) (*S. glanis*) was only slightly susceptible. Striped bass and white perch (*M. americana*), although capable of being infected experimentally, are also only slightly susceptible (Baxa *et al*., 1990; Pasnik *et al*., 2007). In contrast, salmonids, Chinook salmon and rainbow trout, were highly susceptible experimentally (Baxa *et al*., 1990). Plumb and Sanchez (1983) could not infect golden shiners (*Notemigonus chrysoleucas*), largemouth bass or bighead carp (*Aristichthys nobilis*) experimentally and found blue tilapia slightly susceptible. Although strictly considered a fish microbe, *E. ictaluri* has also been isolated from fish-eating birds (great blue heron, great and snowy egret and double crested cormorant) in the USA (Taylor, 1992) during routine monitoring.

Economic importance of the disease

The economic impact of *E. ictaluri* on the channel catfish industry is significant. Wagner *et al*. (2002) reported that ESC and the bacterial disease 'columnaris', caused by *Flavobacterium columnare*, were the two most serious infectious diseases affecting the industry. While not measuring the economic impact of ESC specifically, they emphasized 'that the majority of producers reported them (ESC and columnaris) as the diseases causing the greatest economic impact'. Earlier Plumb and Vinitnantharat (1993) reported that *E. ictaluri* was responsible for US$20–30 million losses annually and Shoemaker *et al*. (2003) estimated that ESC had cost the catfish industry US$60 million annually. *E. ictaluri* poses no infectious threat to non-fish species or to any warm-blooded animals, nor does it present a food safety problem. The temperature limitations under which *E. ictaluri* grows essentially preclude this bacterium from being a pathogen for humans or other warm-blooded animals (Janda *et al*., 1991). However, Skirpstunas and Baldwin (2002) showed that *E. ictaluri* could attach to IEC-6 (rat small intestinal cells) and Henie 407 (human embryonic intestinal epithelium) cells in culture at a low rate, thus indicating that, under some circumstances, this bacterium might invade the intestinal cells of warm-blooded animals but is unlikely to flourish because of its lower temperature intolerance.

Diagnostic methods

Clinical characteristics

Clinical signs of *E. ictaluri* infection are more pathognomonic than most other infectious fish diseases and therefore are helpful in ESC diagnosis. Enteric septicaemia of catfish may be mild, chronic or acute. Diseased fish are listless at the surface, with a 'head-up, tail-down' posture and sometimes spin in circles before death. Other characteristic clinical signs are petechial haemorrhage or inflammation in the skin under the jaw, on the operculum and belly (Fig. 14.5). Haemorrhage often becomes so severe that the skin is bright red. Haemorrhage also occurs at the base of fins. Small white (1–3 mm) depigmented areas appear on the skin and they progress into similar-sized



Fig. 14.5. Channel catfish infected with *Edwardsiella ictaluri*. The upper fish has haemorrhage in the skin under the jaw and isthmus (arrow). The lower fish has white depigmented lesions on the pigmented skin (arrowheads), red ulcerated lesions on the lower gill cover (arrow) and abdominal distension, caused by ascitic fluid in the coelomic cavity.

inflamed cutaneous ulcers. Infected fish also have pale gills, exophthalmia and, sometimes, abdominal distension with ascites that is usually cloudy and/or haemorrhagic and, rarely, clear yellow. The kidney and spleen are hypertrophied, while the spleen is dark red. Inflammation occurs in adipose tissue, peritoneum and intestine, and the liver is either pale or mottled with congestion. One of the most characteristic lesions is an open lesion that develops between the frontal bones of the skull posterior to, or between, the eyes in chronically ill fish, hence the common name 'hole-in-the-head' used in the catfish industry (Fig. 14.6). It should be noted that other bacteria, *A. hydrophila* for example, can cause the same lesion.

Two clinical forms of ESC occur in channel catfish, an acute septicaemia and a chronic encephalitis (Miyazaki and Plumb, 1985; Shotts *et al.*, 1986; Newton *et al.*, 1989). In the acute form of ESC, the bacterium is thought to infect through the intestinal mucosa (Baldwin and Newton, 1993), and then to establish a bacteraemia. The affected fish display petechial haemorrhages around the mouth, on the throat, the abdomen and at the base of the fins. Multifocal, distinct, 2 mm diameter, raised haemorrhagic cutaneous lesions that progress to depigmented ulcers also occur. Anaemia, moderate gill inflammation and exophthalmia are common signs. Internally, haemorrhagic and necrotic foci are scattered in the liver and other internal organs. Haemorrhagic enteritis, systemic oedema, accumulation of ascitic fluid in the body cavity and enlargement of the spleen are non-specific signs. In the chronic form, the bacterium infects the olfactory sacs and migrates along the olfactory nerves to the brain, generating granulomatous inflammation (Morrison and Plumb, 1994). This meningoencephalitis causes abnormal behaviour, with alternating

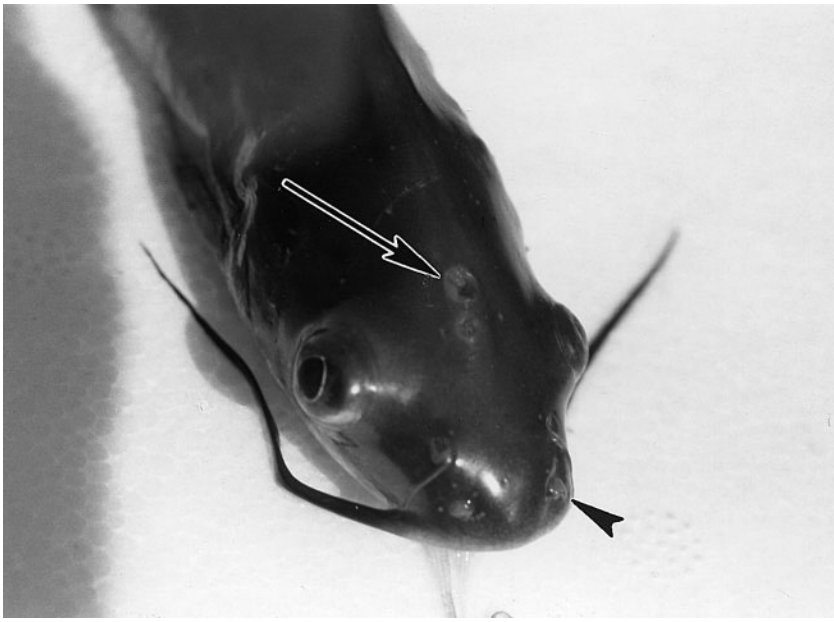


Fig. 14.6. Channel catfish infected with *Edwardsiella ictaluri* exhibiting open lesion (large arrow) in the cranial region and inflamed nares (arrowhead) and exophthalmia typical of chronic infection.

listlessness and chaotic swimming. In the late stages of this disease, swelling develops on the dorsum of the head as the inflammatory process erodes the connective tissue in this region. This swelling ulcerates, exposing the brain.

Culture, immunodiagnostics and molecular techniques

Isolation of the organism, serological tests and/or PCR are essential for confirmation (OIE, 2006). In addition to well-defined biochemical characteristics, there is antigenic homogeneity of the species and serology can differentiate *E. ictaluri* easily from other Enterobacteriaceae (Saeed and Plumb, 1987; Plumb and Klesius, 1988; Bertolini *et al.*, 1990). The *Manual of Diagnostic Tests for Aquatic Animals* provides detailed diagnostic procedures for the identification, detection and screening of *E. ictaluri* (OIE, 2006). Slide agglutination with specific antisera against *E. ictaluri*, fluorescent antibody techniques (FATs), enzyme-linked immunostaining and enzyme-linked immunosorbent assays (ELISAs) have been used to provide confirmatory diagnosis. Characterized specific monoclonal antibodies (MAbs) generally are used in these assays, but polyclonal antisera obtained using formalin-killed bacterins to immunize rabbits according to conventional standards may also be used (Rogers, 1981; Ainsworth *et al.*, 1986; Klesius and Horst, 1991).

E. ictaluri is often isolated from brain and kidney tissue of clinically infected fish on BHI or TSA, but Shotts and Waltman (1990) have developed a selective medium, EIM, which enhances *E. ictaluri* isolation and aids identification, particularly when samples are taken from heavily contaminated environments (OIE, 2006). MacMillan and Santucci (1990) were unable to isolate *E. ictaluri* from the intestine of channel catfish, but Earlix (1995) isolated the organism on EIM from intestines of approximately 50% of clinically infected channel catfish.

The organism forms small, translucent, greenish colonies on EIM (Fig 14.7) that inhibit Gram-positive and most Gram-negative contaminating organisms. Colonies of *E. tarda* have black centres and *A. hydrophila* colonies are brownish and larger, while *P. fluorescens* colonies are blackish and punctate on EIM. Using the biochemical and biophysical characteristics, *E. ictaluri* is separated easily from *E. tarda*, because the former is indole-negative and does not produce H_2S on TSI agar (Table 14.1). Careful observation of culture plates is essential to detect *E. ictaluri*, because of its slow growth and the possible presence of more rapidly growing bacteria, such as *Aeromonas* spp. Commercial identification systems, such as Minitek and API 20E, are not as accurate for *E. ictaluri* as they are for some other fish pathogens (Taylor *et al.*, 1995), although API 20E, FAME and Biolog identified the isolates tested by Klesius *et al.* (2003) as *E. ictaluri*.

Detection of *E. ictaluri* carrier fish when there is no clinical disease may present a problem. However, Mgolomba and Plumb (1992) and Klesius (1992) found significant bacteria in the blood and all organs of fish 65 and 270 days, respectively, after initial exposure to *E. ictaluri* (Fig. 14.8). *E. ictaluri* is also readily phagocytized by macrophages in naive fish, but immunization increases the phagocytic activity (Shoemaker *et al.*, 1997). Indications are that the phagocytized bacteria are not destroyed in these cells, which could lead to a lengthy carrier state and could provide a site of identifying carrier fish (Miyazaki and Plumb, 1985; Klesius *et al.*, 1991; Klesius, 1993). For detection of a carrier state in a healthy population, kidney tissue was homogenized in 0.5% triton-X 100, filtered on to 0.45 µm nitrocellulose and grown on EIM agar medium (Earlix *et al.*, 1996), or homogenized kidney tissue was cultured overnight in liquid EIM and 100 µl of this sample was plated on to BHI agar plates (Klesius, 1992). Earlix *et al.* (1996) used an ELISA and monoclonal antibody in conjunction with kidney tissue homogenization, digestion and filtration as described above to detect an 80% *E. ictaluri* carrier state in asymptomatic channel

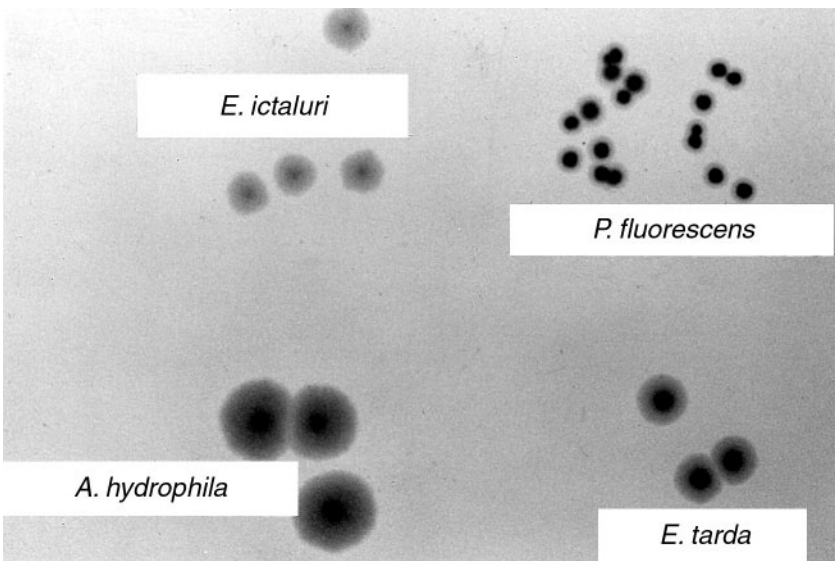


Fig. 14.7. *Edwardsiella ictaluri, E. tarda, Aeromonas hydrophila* and *Pseudomonas fluorescens* on *Edwardsiella* isolation media incubated at 25°C for 48 h (photograph by D. Earlix).

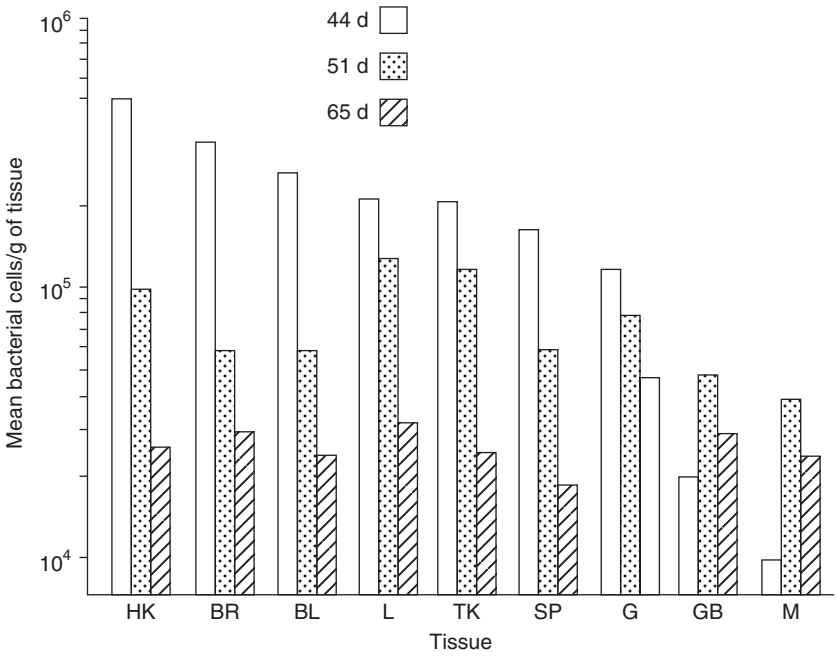


Fig. 14.8. *Edwardsiella ictaluri* isolated from organs and tissues 44, 51 and 65 days after initial exposure to the pathogen. HK, head kidney; BR, brain; BL, blood; L, liver; TK, trunk kidney; SP, spleen; G, gonads; GB, gall bladder; M, muscle (from Mgolomba and Plumb, 1992; reprinted with permission of Elsevier Science Publishers).

catfish. This is compared with 24% carrier-state detection by conventional bacteriological isolation methods. I.p. administration of suspect carrier fish with 0.8 mg/g of Kenalog (triamcinolone acetonide) 2 weeks before attempted culture enhanced the detection of the bacterium (Antonio and Hedrick, 1994). Optimal temperature for incubation is 28–30°C. Application of the Falcon screening test (FAST)-ELISA was utilized by Klesius (1993) to detect *E. ictaluri* antibody rapidly and accurately in adult fish and he proposed that the method could be used to identify possible *E. ictaluri* carrier fish. Implementation of these procedures could be useful in determining *E. ictaluri* carrier populations.

Definitive *E. ictaluri* identification is by using biochemical characteristics and serological identification with specific antiserum agglutination or other serological tests. These include polyclonal and monoclonal antibody in indirect FAT (IFAT) (Ainsworth *et al.*, 1986) and ELISA (Rogers, 1981; Hanson and Rogers, 1989; Klesius, 1993; Earlix *et al.*, 1996). Twenty *E. ictaluri* isolates were positive using FAT and ELISA, while no cross-reactivity was detected with *E. tarda*, *Salmonella* sp. or *A. hydrophila* (Rogers, 1981). Ainsworth *et al.* (1986) also used monoclonal antibodies against *E. ictaluri* in an indirect FAT application for diagnosing *E. ictaluri*. They compared the FAT techniques with bacterial isolation from brain, liver, spleen, anterior kidney and posterior kidney, and 90.3% of the culture-positive fish were also FAT-positive. The greatest discrepancy occurred in the brain samples, but tissues from the spleen were 90% positive by FAT and 85% by culture. All of these studies emphasized the time-saving advantage of immunoassay techniques of 2 h for results versus the 48 h required for culture results. It is also possible to diagnose *E. ictaluri* in the carcasses of dead fish, using an ELISA system (Hanson and Rogers,

1989). Apparently, on death, the bacterium escapes from lysed macrophages and uses the nutrients of these to proliferate, thus providing a large number of organisms. A rapid IFAT was developed that allowed simultaneous detection of *E. ictaluri* and *F. columnare* (Panangala *et al.*, 2006a). This IFAT test compared favourably in sensitivity and specificity with standard microbiological culture.

Serum agglutination, passive haemagglutination, complement-dependent passive haemolysis, indirect immunofluorescence, agar gel immunodiffusion and agglutination with fractionated immunized fish sera were used to detect humoral antibody to LPS of *E. ictaluri* (Saeed and Plumb, 1987). All tests were sensitive to the LPS antibody, with the complement-dependent haemolysis titres being the highest (average titres of 1:2360). Waterstrat *et al.* (1989) found a close relationship of antibody titres measured by optical density and corresponding agglutination titres to *E. ictaluri* in channel catfish. Using the FAST-ELISA system employing a monoclonal antibody against an immunodominant epitope of *E. ictaluri*, Klesius *et al.* (1991) found no cross-reactivity with sera from channel catfish immunized experimentally against *E. tarda* or *A. hydrophila*. The ELISA system was further refined so that either *E. ictaluri* antigen or antibody in fish could be detected in 30 min. Time is usually of the essence in diagnosing clinical *E. ictaluri*; hence, the rapid serological techniques should be used in conjunction with bacterial isolations (Plumb, 1999a).

Molecular techniques

Molecular techniques to detect *E. ictaluri* have been developed. Assays based on PCR amplification of structural RNA sequences from bacterial colonies and direct sequencing of the products are being adapted by several diagnostic bacteriology laboratories and some of these assays are commercially available from MicroSeq, Applied Biosystems.[1] Species confirmation can be done by amplifying and sequencing the 16S portion of the ribosomal RNA operon and comparing the sequence with GenBank accession AF310622. Bilodeau *et al.* (2003) developed a specific real-time PCR method that detected *E. ictaluri* in blood and tissues. However, the limitations of PCR should be realized (i.e. the assay can be positive when non-viable bacteria are present) when interpreting data generated using these methods. A multiplex-PCR method was developed for simultaneous detection of *E. ictaluri*, *F. columnare* and *A. hydrophila* (Panangala *et al.*, 2007).

Genome structure and transcription

Genome, gene expression, proteins and glycoproteins

The molecular composition of the polymeric structure in *E. ictaluri* remains largely unknown. Its genome is a single circular chromosome. *E. ictaluri* isolate 93–146, which was isolated originally from an ESC outbreak in a commercial catfish pond in Louisiana (USA), was used to determine the *E. ictaluri* genome sequence. Two plasmids (5.6 and 4.8 kb) are consistently present in channel catfish isolates (Newton *et al.*, 1988; Lobb *et al.*, 1993) and both plasmid sequences have been published (Fernandez *et al.*, 2001).

Panangala *et al.* (2008) described the flagellin gene sequences and their organization. Four flagellin genes are arranged in tandem within 6 kb in the *E. ictaluri* genome. The second, third and fourth genes are separated by less than 300 nt. The first and second flagellin genes are separated by 1.1 kb, which include a 172-codon ORF with homology to the amino terminal portion of a mutator-type transposase distinct from the *E. ictalur*i TnpA. The predicted amino acid sequences of all four genes are similar in the N-terminal (AA 1–160) and C-terminal (last 74 AA) portions and are divergent in the central portion of the proteins. The proteins encoded by the first three flagellin genes are more similar to each other (88–90% AA identity) than to the protein encoded by the fourth flagellin gene (76–78% AA identity). The predicted sizes of the encoded proteins range between

36 and 37.5 kDa. BLAST analysis of GenBank sequences showed that all four flagellin AA sequences were more similar to those of *Serratia marcescens* (72–74% identity) than to those of *E. tarda* (equal to or less than 62% identity).

DNA hybridization studies indicate that *E. ictaluri* is related most closely to *E. tarda*, with a relative binding ratio of 56–60% at 60°C. *E. ictaluri* had a relative binding ratio of 31% to *E. coli* at 60°C. The G + C content of *E. ictaluri* was estimated at 53% by buoyant density centrifugation (Hawke *et al.*, 1981).

Pathogenesis and immunity

Disease progression and histopathology

E. ictaluri infects fish by several routes. Waterborne bacteria can invade the olfactory organ via the nasal opening and migrate into the olfactory nerve, then into the brain meninges and finally to the skull and skin (Miyazaki and Plumb, 1985; Shotts *et al.*, 1986; Morrison and Plumb, 1994). Injury to the nasal passage includes loss of sensory cilia and microvilli from the olfactory mucosal surface within 1 h of exposure to *E. ictaluri*. By 24 h, the olfactory receptors and supporting cells degenerate (Fig. 14.9); electron microscopy confirmed the presence of *E. ictaluri* on the mucosal surface and within the epithelium. Host leucocytes migrate through the olfactory epithelium into the lamellar lumen and phagocytize the bacterium (Morrison and Plumb, 1994). With regard to the attachment mechanism of *E. ictaluri*, Wolfe *et al.* (1998) showed that bacterial lectins were instrumental in this attachment by utilizing specific sugar residues, specifically D-mannose, *N*-acetylneuraminic acid and L-fucose, in the nasal mucosa.

E. ictaluri apparently colonizes capillaries in the dermis and causes necrosis and depigmentation of the skin of infected fish. In the intestine, *E. ictaluri* enters the blood through the intestinal wall and is engulfed by macrophages, resulting in septicaemia (Shotts *et al.*, 1986; Newton *et al.*, 1989). Channel catfish exposed to *E. ictaluri* via oral infection developed enteritis, hepatitis, interstitial nephritis and myositis within 2 weeks of infection. Francis-Floyd *et al.* (1987) described gastrointestinal lesions, including petechiae or ecchymoses, in the mucosa of the gastrointestinal tract and intestinal distension associated with gas production. The gill is also a primary site of *E. ictaluri* invasion. Using radiolabelled *E. ictaluri*, Nusbaum and Morrison (1996) demonstrated that, during immersion, the organism colonized the gill epithelium in large numbers in 2–72 h. Bacterial numbers then increased rapidly in the liver and less rapidly in the trunk kidney, gut and brain. Heat-killed radiolabelled bacteria did not appear to cross the gill epithelium membrane (Plumb, 1999a).

Histopathologically, the trunk kidney and spleen are the most severely affected organs in channel catfish, both of which are necrotic, while the liver is oedematous and necrotic (Fig. 14.10) (Areechon and Plumb, 1983). Proliferation occurs in interlamellar tissue in gills (Jarboe *et al.*, 1984; Miyazaki and Plumb, 1985; Shotts *et al.*, 1986). Also, a mild focal inflammatory cell infiltration, necrosis and granulomatous inflammation take place in the underlying musculature in areas where the epidermis is missing. Intact *E. ictaluri* cells are also seen in macrophages, similar to *E. tarda* (see Fig 14.3). Histological examination reveals a systemic infection of all organs and skeletal muscles, with the most severe changes being diffuse interstitial necrosis of the anterior and posterior kidney. Focal necrosis is also generally seen in the liver and spleen.

Newton *et al.* (1989) exposed channel catfish experimentally to 5×10^8 cfu *E. ictaluri*/ml, and 93% of the affected fish developed acute ESC and 7% developed chronic infection. Acute disease was characterized grossly by haemorrhage and ulceration and microscopically by enteritis and olfactory sacculitis at 2 days post-exposure, followed by hepatitis and dermatitis. Chronic ESC, seen at 3–4 weeks post-exposure, was characterized by dorsocranial swelling and ulceration, granulomatous inflammation and

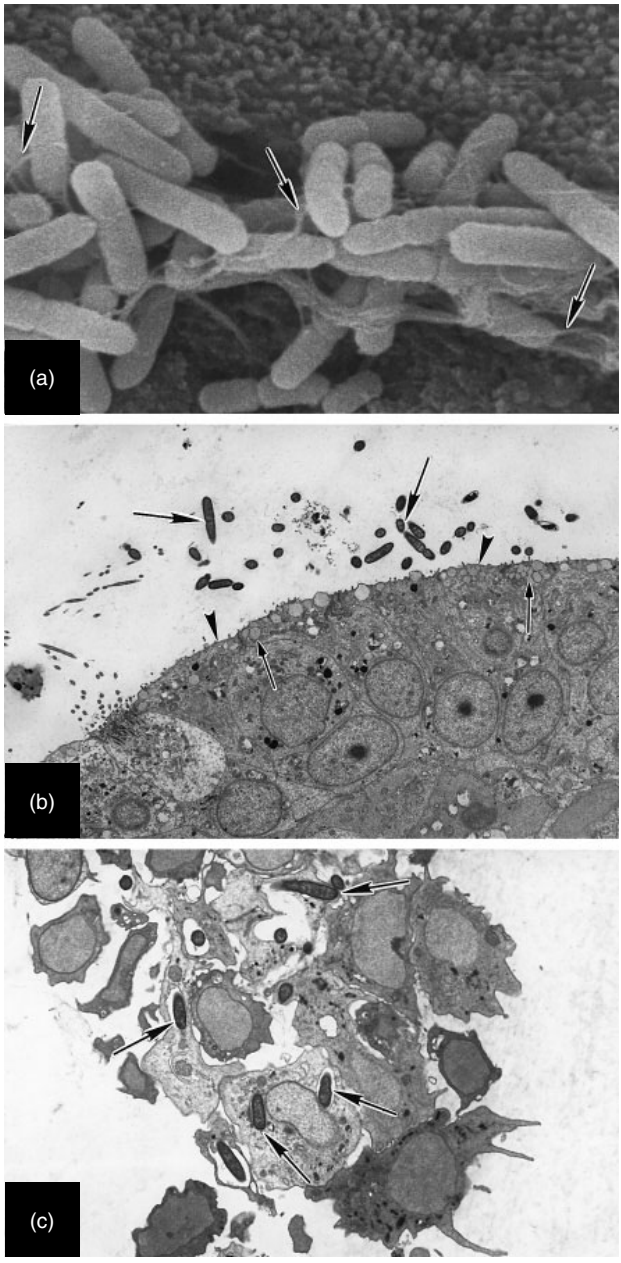


Fig. 14.9. Electron micrographs of *Edwardsiella ictaluri* in olfactory organ of channel catfish. (a) Scanning electron micrograph of the olfactory mucosal surface following direct experimental *E. ictaluri* infection with a cluster of bacteria attached to the epithelial surface. Note the fine filamentous processes extending from *E. ictaluri* (arrows) (× 13,500); (b) transmission electron micrograph of the olfactory mucosal surface 1 h following direct experimental *E. ictaluri* infection. The bacteria (large arrows) are close to the epithelial surface (arrowhead), which has no cellular cilia but has increased secretory vesicles of host mucosal cells (small arrows) (× 2565); (c) transmission electron micrograph showing several catfish leucocytes in the interlamellar space of the olfactory rosette. A number of *E. ictaluri* can be seen within cellular phagosomes (arrows) (× 4590) (photographs courtesy of E.E. Morrison and K.G. Wolfe).

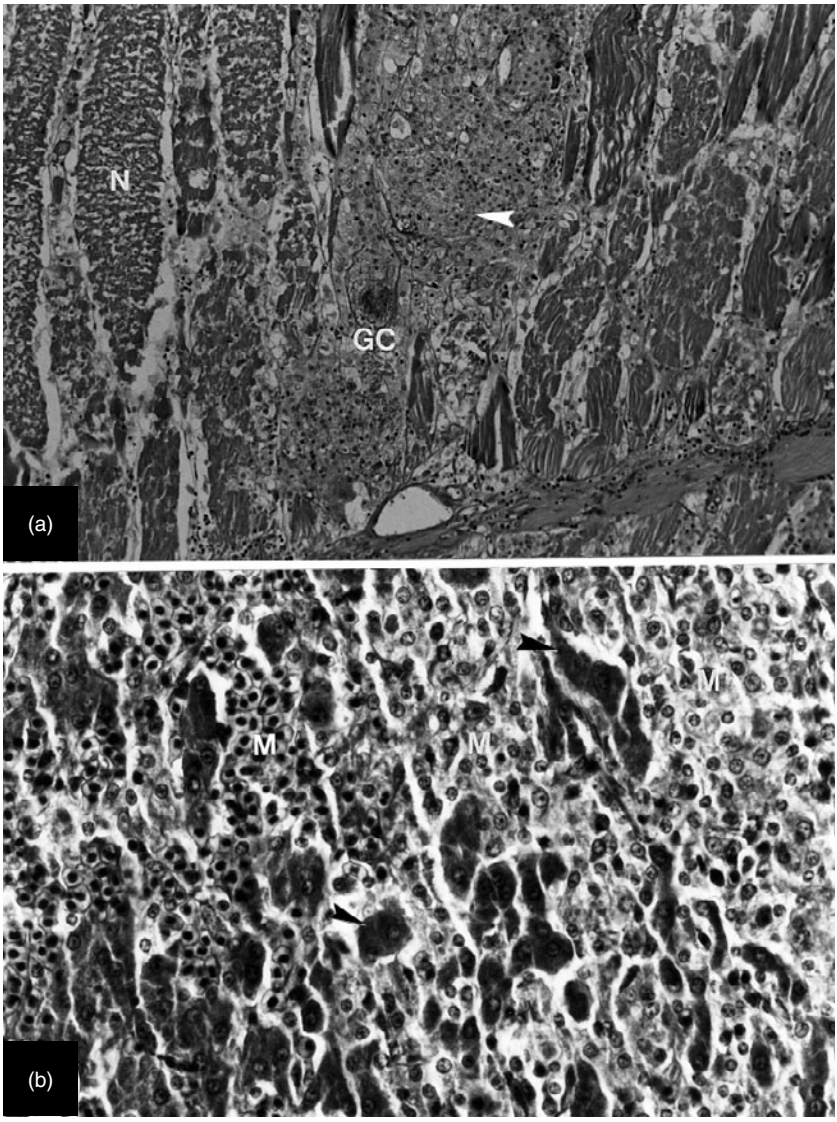


Fig. 14.10. Paraffin sections of tissue from a channel catfish infected with *Edwardsiella ictaluri*. (a) Necrotic skeletal muscle (N) with infiltrating macrophages (arrow) typical of granulomatous inflammation. A giant cell (GC) is present within the accumulation of macrophages (H & E, ×450); (b) focal accumulation of macrophages (M) in the liver. Within this lesion, the macrophages have displaced most of the liver tissue, but scattered hepatocytes (arrows) are still present (H & E, ×1000) (photograph by A. Goodwin).

meningoencephalitis of the olfactory bulbs, olfactory tracts and olfactory lobes of the brain. The granulomatous inflammation in chronic *E. ictaluri* infection is a key histopathological characteristic of ESC (Fig. 14.10). Skeletal muscle becomes necrotic, with infiltration of macrophages, while internal organs, especially the liver, have normal tissue displaced by macrophages (Plumb, 1999a).

Disease mechanism and virulence factors

The pathogenic mechanism of *E. ictaluri* infection in channel catfish is not understood fully. Janda *et al.* (1991) demonstrated that, in contrast to *E. tarda* infection, the bacteria did not invade HEp-2 cell monolayers at 35°C, nor did they produce cell-associated haemolysin or siderophores. In examining ECP associated with the pathogenesis of *E. ictaluri*, Stanley *et al.* (1994) found a fibrillar network connecting virulent cells that could aid in attachment. Virulent isolates had greater amounts of capsular material and surface proteins, and they demonstrated a greater ability to degrade chondroitin than did avirulent cells. These authors reported no clear correlation between haemolytic activity and virulence.

Immunity

Early studies examining immunity to *E. ictaluri* suggested that antibody was important and responsible for protection following infection (Vinitnantharat and Plumb, 1993; Thune *et al.*, 1994). Other studies have demonstrated that immunity to ESC is cell mediated in nature (Antonio and Hedrick, 1994; Klesius and Sealey, 1995; Klesius and Shoemaker, 1997; Thune *et al.*, 1997b). Initial encounter with *E. ictaluri* signals innate immune mechanisms including antimicrobial peptides, lysozyme, toll-like receptors (TLR) (Bilodeau *et al.*, 2006; Baoprasertkul *et al.*, 2007), complement (Nonaka and Smith, 2000), acute phase proteins (Peatman *et al.*, 2008), cytokines such as TNF-α (Zou *et al.*, 2003) and natural killer cells (Shen *et al.*, 2004). The initial recognition is probably by the TLRs that react with lipopolysaccharide (LPS) to signal *E. ictaluri* presence. A receptor on macrophages that recognizes bacterial LPS (a CD 14 homologue) enables the phagocytic process to occur. *E. ictaluri* survive and replicate within the host macrophage (Booth *et al.*, 2006), which results in induction of cellular immunity (CD4+ and CD8+ T-cell responses) against intracellular infections (Seder and Hill, 2000). Recent studies have demonstrated the relevant major histocompatibility complex (MHC) class I (Antao *et al.*, 2001) and class II molecules (Goodwin *et al.*, 2000) in fish. Presentation of antigen with the correct MHC allows for the response and recognition by the appropriate subpopulations of T- and B-cells. Seder and Hill (2000) suggest that intracellular pathogens induce Th1 and CD-8 T-cell responses. Recent transcriptome analysis of gene expression in catfish liver tissue suggests that on exposure to live bacteria, MHC class I genes, along with other acute phase response genes, are upregulated, suggesting active antigen processing and presentation (Peatman *et al.*, 2008). Induction of MHC class I genes suggests a CD-8 T-cell response following infection with intracellular *E. ictaluri*. Subsequent stimulation of a subpopulation of T-cells may induce interferon gamma (Milev-Milovanovic *et al.*, 2006) production that can mediate intracellular pathogen destruction (Seder and Hill, 2000).

Epidemiology, treatment and control

Epidemiology

Mortality in naturally infected channel catfish populations varies from less than 10% to over 50%. It occurs in juvenile as well as food-sized fish, and under all types of cultural conditions (including ponds, raceways, recirculating systems and cages). Few fish diseases occur without some environmental stressor preceding the infection, but *E. ictaluri* can probably cause disease independent of stressors. This is not to suggest that adverse environmental conditions do not influence the severity of infection, because Wise *et al.* (1993a) showed that, when channel catfish were stressed by confinement in tanks prior to *E. ictaluri* exposure, there was 97% mortality in stressed fish and 77% mortality in non-stressed fish. To illustrate the effects of stress further, Ciembor *et al.* (1995) netted and handled channel catfish and then exposed them to waterborne *E. ictaluri*, leading to a mortality of 53% in stressed fish and 16% in unstressed fish. It was also shown by Plumb

et al. (1993b) that stocking density in ponds might affect susceptibility to *E. ictaluri*.

ESC is considered a seasonal disease, occurring primarily in late spring to early summer and again in the autumn in regions where channel catfish are normally cultured (Fig. 14. 11). Acute outbreaks of ESC occur within a limited temperature range. This pattern generally coincides with, but is not confined to, water temperatures of 18–28°C. Low-level mortality due to ESC can occur in carrier populations outside this temperature range. Other environmental factors (poor water quality, high stocking density and other stressors) predispose the host to ESC.

Francis-Floyd *et al*. (1987) demonstrated that the highest mortality in experimentally infected channel catfish fingerlings was at 25°C, lower at 23 and 28°C and no deaths at 17, 21 or 32°C. Several experiments have further substantiated the temperature preference of ESC. Baxa-Antonio *et al*. (1992) used immersion of channel catfish in a bath containing *E. ictaluri* to show peak mortality at 25°C (98%) and lower mortality at 20°C (47%), 30°C (25%), 35°C (4%) and 15°C (0%). The effect of 25°C on clinical ESC was further demonstrated by Plumb and Shoemaker (1995) using a naturally infected population of channel catfish in which 10% were culture-positive for *E. ictaluri* while being held at 15°C. When these fish were elevated to 25°C, 77% mortality occurred due to *E. ictaluri*, which was significantly higher than that at 18 or 30°C (10 and 23%, respectively). In spite of the compelling experimental data that implicate a mid-20°C optimum temperature, an increasing prevalence of ESC cases submitted

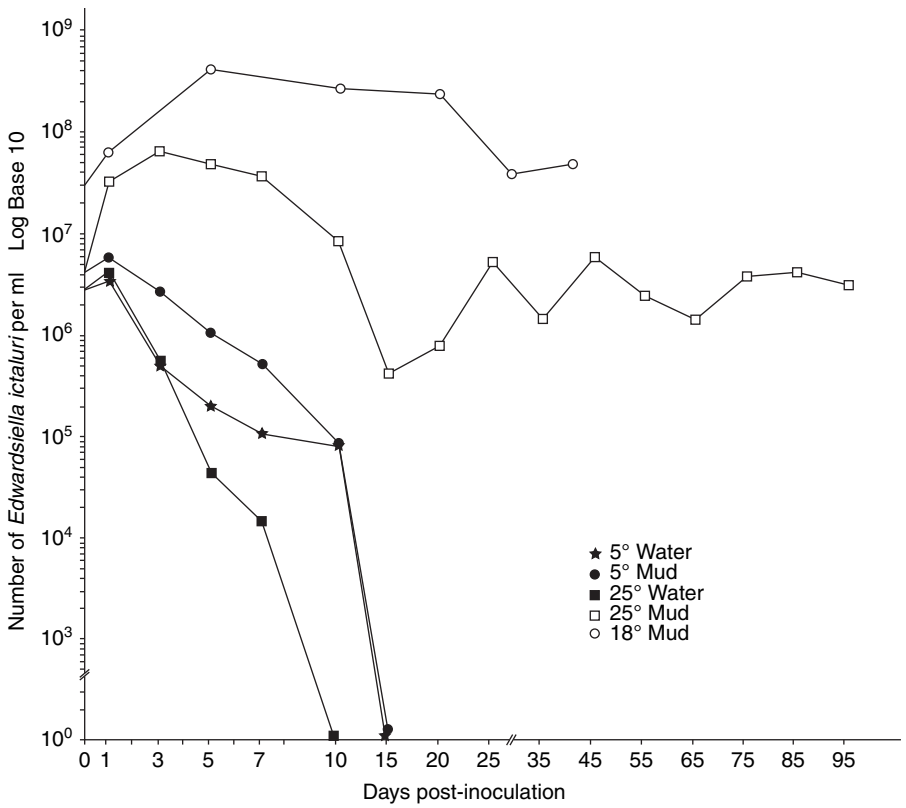


Fig. 14.11. Seasonal occurrence of *Edwardsiella ictaluri* showing greatest prevalence of disease in May, June, September and October, when average water temperatures are 20–27°C.

to diagnostic laboratories during winter and summer has been noted, indicating possible adaptation of *E. ictaluri* to a broader temperature range (J.A. Plumb, unpublished). During the early years following the discovery of ESC, relatively few outbreaks of the disease were reported, but the number of *E. ictaluri* isolates soon began to occur at an alarming rate. In 1981, there were 47 outbreaks diagnosed in the south-eastern USA and in 1985, there were 1420 diagnosed outbreaks, thus accounting for 28% of all reported fish-disease cases in the region (A.J. Mitchell, Fish Farming Experiment Station, Stuttgart, Arkansas, USA, 1996, personal communication). In 1988, there were 1605 documented reports of ESC (30.4% of reported fish diseases); however, the prevalence levelled during 1990 and 1991 and since then has remained constant. In a survey by Wagner *et al.* (2002), it was found that 78.1% of all channel catfish operations and 42.1% of production ponds experienced columnaris and/or ESC, or a combination of the two, with no distinction being made between the two. Average losses were from 90 to 900 kg of fish per disease episode.

Transmission control

Best management practices of cultured fish to reduce the effects of ESC are important and include minimizing stressors, preventing overcrowding, using proper feeds but not overfeeding, maintaining high water quality and removing dead fish as soon as possible (Plumb, 1999b). In addition to the application of the best management practices, the use of strains of channel catfish that are less susceptible to *E. ictaluri* or utilizing channel catfish–blue catfish hybrids shows promise of providing possible culture animals on farms where *E. ictaluri* is endemic (Wolters and Johnson, 1994; Wolters *et al.*, 1996). These practices are combined with judicious application of chemotherapy for overt disease and preventive vaccination when vaccines are available.

Although there is a tendency for the aquaculturist to 'do something' when ESC strikes, it has been suggested that discontinuing feeding of fish every day may be as good a management decision as feeding antibiotic-medicated feed. It has been shown that depriving feed and feeding medicated feed (sulfadimethoxine–ormetoprim) every third day resulted in the highest survival of *E. ictaluri* infected fish, compared with daily feeding with a normal ration (Wise and Johnson, 1998). Skipping 1 or 2 days between feeding was also better than a daily feeding regime. Discontinuing feeding when ESC occurs has been adopted by many channel catfish farmers in place of feeding a medicated diet, generally with satisfying results. However, Lim and Klesius (2003) evaluated feeding regimens in laboratory trials and their results suggested that fish fed daily or fed every other day were more resistant than non-fed fish. In an attempt to evaluate the value of winter feeding on ESC susceptibility in the spring, Kim and Lovell (1995) and Okwoche and Lovell (1997) demonstrated that production-size channel catfish deprived of feed during winter were more resistant to *E. ictaluri* in the spring than those fish that had received normal or partial feeding during the winter months.

Diet may be important in altering susceptibility of channel catfish to *E. ictaluri*. Channel catfish fed diets deficient in zinc had 100% mortality, compared with 25–30% mortality for those fish fed 15–30 mg of zinc (Paripatananont and Lovell, 1995). Other nutritional factors may affect susceptibility of channel catfish to *E. ictaluri*, because the addition of 60 mg or more of vitamin E in the channel catfish diet increased agglutinating antibody titres and enhanced the ability of macrophages to phagocytize virulent bacteria (Wise *et al.*, 1993b). In contrast, Tyler and Klesius (1994) showed that the injection of squalene, an oil-based adjuvant, actually increased the susceptibility of channel catfish to *E. ictaluri*. Similarly, Stanley *et al.* (1995) showed that a normally immunoenhancing compound, an extract from the tunicate (*Ecteinascidia turbinate*), had an adverse effect on disease susceptibility when injected intraperitoneally into channel catfish.

Channel catfish are less susceptible to *E. ictaluri*, and possibly other pathogens, in

water with salinity of up to 4000 mg/l (G. Whitis, Aquaculture Extensionist, Alabama Fish Farming Center, Greensboro, Alabama, USA, 1995, personal communication). In view of this observation, Plumb and Shoemaker (1995) exposed an *E. ictaluri* carrier population (about 10% prevalence in 15°C water) of channel catfish to waters containing 0–3000 mg/l NaCl and raised the temperature to 25°C. Percent mortality in 0 and 100 mg NaCl/l was 95–100 and percent mortality in the populations transferred to water with over 1000 mg NaCl/l was 17–42. It is apparent that many factors, including genetics, nutrition and environmental quality, affect the susceptibility of channel catfish to *E. ictaluri* infection, the underlying reasons for which are poorly understood.

Chemotherapy

ESC may be controlled through chemotherapy and/or prophylactic measures. The most common antimicrobial treatments are oral application of the potentiated sulfonamide, sulfadimethoxine–ormetoprim, or oxytetracycline, but plasmid-mediated resistance to these antibiotics does occur (Cooper *et al.*, 1993). Two drugs, oxytetracycline (Terramycin) and sulfadimethoxine–ormetoprim (Romet-30), are approved by the US Food and Drug Administration (FDA) for *E. ictaluri* infections in the USA (Schnick *et al.*, 1989). Both are incorporated into manufactured feed; oxytetracycline is fed at 50–75 mg/kg of fish/day for 12–14 days, followed by a 21-day withdrawal period. Sulfadimethoxine–ormetoprim is also fed at 50–75 mg/kg of fish for 5 days, followed by a 3-day withdrawal for catfish. The importance of an early diagnosis cannot be overemphasized in ESC, because successful therapy depends on expedience and immediate application of medicated feed. The sulfadimethoxine–ormetoprim may create palatability problems if the concentration in the feed is too high, and the fish will stop feeding. To prevent palatability problems, Johnson and Smith (1994) suggested that the concentration of drug in the feed be reduced by at least half and the pellet made smaller and fed at 3% of body weight. Florfenicol (Aquaflor) has been approved for ECS treatment recently. Sarafloxacin, a quinolone, was shown to be effective for treating *E. ictaluri* infections of channel catfish at 10 mg/kg of fish/day for 5 or 10 days under experimental conditions (Plumb and Vinitnantharat, 1990). The superiority of the 10-day feeding of sarafloxacin was demonstrated by Thune and Johnson (1992), because 15 days after cessation of treatment, the number of carrier fish in the 5-day feeding group was nearly twice that of the 10-day feeding group. Field trials by Johnson *et al.* (1992, 1993) demonstrated the efficacy of sarafloxacin on *E. ictaluri* infected channel catfish held in either ponds or cages. However, the FDA has not approved sarafloxacin for *E. ictaluri* infections.

Waltman and Shotts (1986b) screened 118 isolates of *E. ictaluri* for susceptibility to 37 antimicrobials, including oxytetracycline and sulfadimethoxine–ormetoprim. They found no evidence of resistance to either of these antibiotics; however, there have been reports of resistance to both (Plumb *et al.*, 1995; P. Taylor, US Fish and Wildlife Service, Marion, Alabama, 1995, personal communication). The increase in resistance to drugs is exacerbated by improper use of the antibiotics by feeding medicated feed when it is not necessary, feeding at an incorrect rate or applying the medicated feed for too long, too often or for too short a time. The increased resistance could also be plasmid-induced, as was suggested by Waltman *et al.* (1989). Many producers are now focusing on alternative methods to reduce losses, relying on management to reduce stress in fish, the cessation of feeding when ESC-induced losses are detected (Wise and Johnson, 1998) and on vaccination (Klesius and Shoemaker, 1999; Shoemaker *et al.*, 1999; Wise *et al.*, 2000; Klesius *et al.*, 2004; OIE, 2006).

Vaccinology

E. ictaluri is a strong immunogen, especially when injected, and therefore is an excellent candidate for vaccine development (Vinitnantharat and Plumb, 1992). Antibody titres against *E. ictaluri* are measured by agglutination or ELISA, but lower titres do not

correlate necessarily with protection (Klesius and Sealey, 1995). Vinitnantharat and Plumb (1993) showed that channel catfish that survived a natural epidemic of ESC had varying levels of antibody and that those fish with high titres (>1:1024) suffered 6.5% mortality when challenged by injection, compared with 25 and 51% in fish with medium titres of 1:256–512 and low titres of 0–1:128. Most of the *E. ictaluri* vaccination studies discussed below do not elicit humoral antibody titres that approach even the medium range noted above, which therefore may help explain why some experimental vaccinations have been unsuccessful in providing protection against ESC.

The kinetics of the immune response of channel catfish to i.p. injection with either cell extracts or whole-cell preparations of *E. ictaluri* and held in cages at 20–30°C showed peak titres of 1:200–1:2000 2–8 weeks following injection (Vinitnantharat and Plumb, 1992). Channel catfish at 25°C produced a rapid immune response to whole-cell bacterins; however, if fish were vaccinated, held at 25°C for 4 days and then the temperature reduced to 12°C, the agglutination titre was higher and had greater longevity (Plumb *et al.*, 1986). These fish also showed greater antibody titres in the memory response than in the primary response, when given a second injection. Specific *E. ictaluri* antibody following immersion in a live bacterial bath can occur as quickly as 5 days after exposure (Klesius and Sealey, 1995). Significant antibody stimulation occurred 14–21 days after exposure and began to decline at 28 days. These fish developed a second response when re-exposed 84 days after initial contact, but this response was not greater than the initial response. After the initial exposure, the fish were protected against subsequent exposure to virulent *E. ictaluri*.

Immersion in a 1:10 dilution of the killed bacterin is the optimum concentration (Morsey, 1988; Vinitnantharat and Plumb, 1992) and length of immersion is 0.5–2 min or more, but the longer the exposure, the better the response. Laboratory studies indicate that *E. ictaluri* vaccine incorporated into the feed is immunogenic and may serve as a booster vaccination. Plumb and Vinitnantharat (1993) combined immersing juvenile channel catfish in 1:10 formalin-killed bacterin and then feeding an encapsulated preparation in the feed for two 5-day periods, with a 10-day non-vaccination period in between. Six months later, agglutinating antibody titres in the immersion- plus oral-vaccinated fish averaged about 1:1700, compared with 1:931 in the non-vaccinated fish. The immune response may have been aided by natural exposure to *E. ictaluri*, but no clinical ESC was detected. When subsamples of fish were challenged with *E. ictaluri*, the survivals were 42.7% in non-vaccinated controls, 56.3% in immersion vaccinates and 70.8% in immersion plus oral vaccinates. Accordingly, exposure of channel catfish to a formalin-killed *E. ictaluri* bacterin by immersion may provide marginal protection, but immersion plus oral application increases protection. The concentration of encapsulated ESC vaccine in the feed should be at least 0.5%, with 1% vaccine being optimum, although 10% vaccine was not immunosuppressive (Plumb *et al.*, 1994).

In a field study involving 12,500 to 2.4 million juvenile channel catfish on each of four different farms, Plumb *et al.* (1993a) investigated the practical application of vaccinating against *E. ictaluri*. There was no experimental laboratory challenge, but the overall survival of non-vaccinated fish was 43.6%, a single immersion was 56.7%, a double immersion was 64.2% and immersion plus oral application resulted in 68.8% survival. Similar results were reported by Thune *et al.* (1994), but they found one immersion/oral vaccination regime to have resulted in very high survival (95%), compared with immersion vaccinates (23.3%) and non-vaccinates (38.3%). In a study of channel catfish vaccinated by immersion followed by the 5- to 10- to 5-day oral booster regime and non-vaccinated fish stocked at 6000, 12,000 and 24,000/ha in 0.04/ha ponds, vaccinated fish at the two lower densities experienced 20% higher survival ($P<0.05$) than non-vaccinated fish (Plumb *et al.*, 1993b). Vaccination induced no benefit at the higher stocking density.

Several studies have examined the potential of fractionating *E. ictaluri*, followed by isolating and purifying an immunodominant antigen (Plumb and Klesius, 1988; Klesius and Horst, 1991; Vinitnantharat *et al.*, 1993). It appears that proteins with a molecular mass of 36 kDa and 60 kDa are primary immunodominant antigens in the cell membrane, which provide protection to channel catfish when injected (Vinitnantharat *et al.*, 1993). The 36 kDa protein is not lost by cells when cultured for up to 30 passages on media. Saeed and Plumb (1986) demonstrated that *E. ictaluri* LPS was an excellent antigen when injected along with adjuvant, but immersing fish in LPS was not immunogenic. All immersion and/or oral vaccination studies with *E. ictaluri* bacterins and encapsulated products have not been successful. One possible reason for these failures is that the fish may not absorb the killed antigen in the digestive tract and therefore it does not reach immunogenic tissues (Nusbaum and Morrison, 1996). Also, humoral antibody may not be produced and, even if present, may not be protective (Klesius and Sealey, 1995).

Until recently, experimental vaccines have been either formalin-killed, whole-cell bacterins, sonicated cell preparations or cell extracts, such as LPS or immunodominant antigens, but attenuated preparations of *E. ictaluri* are now receiving some attention. Shoemaker *et al.* (1997) and Shoemaker and Klesius (1997) strongly suggested that an effective *E. ictaluri* immersion vaccine required a live bacteria to mediate the cell-mediated immunity, which killed preparations failed to do. They demonstrated that channel catfish that survived exposure to a live, moderately virulent *E. ictaluri* isolate were 100% protected against subsequent exposure to virulent *E. ictaluri*, but channel catfish vaccinated by immersion or orally with killed bacterins had 68 and 50% survival, respectively. In support of the efficacy of the exposure to live bacteria, the *in vitro* bactericidal activity by peritoneal macrophages from live-cell vaccinates was significantly greater ($P<0.05$) than bactericidal activity of macrophages from the other groups of vaccinates (Shoemaker and Klesius, 1997). The protection afforded by initial exposure was correlated to cell-mediated immunity rather than humoral antibody. An irreversible attenuated *E. ictaluri* would be required for live vaccine preparations.

The possibility of protecting channel catfish populations by vaccination has been reviewed by Plumb (1988), Thune *et al.* (1997a), Shoemaker *et al.* (2003) and Klesius *et al.* (2001, 2004). *E. ictaluri* is known to induce an antibody response after natural disease or immunization. A commercial vaccine consisting of an inactivated bacterin was licensed provisionally in the USA against ESC and marketed in 1991. The effectiveness of this vaccine is low and it is no longer available. Thune *et al.* (1997b) attempted to vaccinate young fish (12 days posthatch) with a formalin-killed vaccine by immersion and immersion followed by oral booster. Results of this study demonstrated no protective effect in young fry by either vaccination regime. Age-related factors and the induction of a cellular immune response are of critical importance in inducing strong anti-*E. ictaluri* defences. Studies show that catfish fry under 3 weeks of age are immunologically unresponsive to *E. ictaluri* (Petrie-Hanson and Ainsworth, 1999), and this is thought to be due to poorly developed lymphoid organs in the young fish (Petrie-Hanson and Ainsworth, 2001).

New attenuated agents show promise in inducing more effective immune responses (Cooper *et al.*, 1996; Lawrence *et al.*, 1997; Klesius and Shoemaker, 1999; Thune *et al.*, 1999). Cooper *et al.* (1996) described an *E. ictaluri* isolate that was modified genetically to be chondroitinase-negative, and preliminary studies suggested that when this mutant was injected into channel catfish, it provided protection against pathogenic *E. ictaluri*. Lawrence *et al.* (1997) and Thune *et al.* (1999) described auxotrophic mutants of *E. ictaluri* (purA and aroA, respectively) as potential attenuated *E. ictaluri* vaccines. These potential vaccine strains failed to persist for greater than 72 h following immersion exposure and, if delivered at high numbers, induced clinical ESC. Klesius and Shoemaker (1999) developed a vaccine strain (RE-33) by passage on rifampicin, an

antibiotic that caused changes in the LPS of Gram-negative bacteria (Schurig *et al.*, 1991). Reversion to virulence and back passage studies demonstrated this vaccine isolate was safe for immersion vaccination of young channel catfish (Klesius and Shoemaker, 1999; Shoemaker *et al.*, 1999). This mutant is licensed by USDA-APHIS-CVB and is commercially available (Klesius and Shoemaker, 1999) and when administered to 7- to 12-day-old fry, persists long enough to induce protective immunity (Shoemaker *et al.*, 1999; Wise and Terhune, 2001). Apparently, RE-33 is effective because macrophages and antigen-presenting cells are present at this early life stage and/or the live vaccine persists long enough (i.e. at least 12 days) for the immune system to become responsive (Shoemaker *et al.*, 2002). This vaccine strain has been characterized thoroughly and has been shown to lack high molecular weight bands of LPS by immunoblot when compared with the parent isolate (Arias *et al.*, 2003). The attenuation is due to the modification in the LPS profile. About 25% of all catfish fry and/or fingerlings produced in south-eastern USA since the vaccine AQUAVAC-ESC™ became available in 2000 have been immunized. Field trials by Intervet, Inc. suggest that the vaccinated fish consumed more feed, grew faster and resulted in larger fingerlings. Use of the vaccine (even taking into account the cost of vaccination) resulted in 13% improved survivability and an improved return of about US$1700/acre compared with non-vaccinated fish. More recently, Shoemaker *et al.* (2007) demonstrated the effectiveness of a bivalent vaccine against both *F. columnare* and *E. ictaluri*. Additional research in developing genetically resistant strains of catfish shows promise (Wolters and Johnson, 1994; Camp *et al.*, 2000) and may help to reduce losses due to ESC. Wise *et al.* (2000) reported that some channel catfish families were more responsive to vaccination than others. Development of strains of catfish more responsive to vaccination may be a good approach to ESC management, because this strategy relies on stimulation of the adaptive immune response.

Conclusions and Recommendations for Future Studies

E. tarda and *E. ictaluri* both cause economically important diseases in aquaculture, but their epidemiological implications and manifestation need elucidation. Since neither bacterium is a strict obligate pathogen, we need to learn how and where they survive in fish and the environment. Knowledge of how cultural practices enhance their spread, particularly *E. ictaluri* throughout the catfish industry, would be most helpful. The role and effect of climate, water quality and environmental conditions, fish genetics, nutrition and other management practices on ES and ESC should be investigated further and would aid in better management of these diseases. Why these bacteria are more pathogenic at certain temperatures and the nature of their primary pathogenesis are other areas worthy of study. To understand the pathogenesis of these organisms and their mechanism of attachment and invasiveness to the host and the identification of pathogenic components could enhance the utilization of preventive measures. Chemotherapeutic control of *E. tarda* and *E. ictaluri* is a problem, because they have transmissible R plasmids which enhance antibiotic resistance. Educating and encouraging aquaculturists to practise health maintenance disease prevention and to apply drugs properly and only when necessary will slow the evolution of resistance to commonly used drugs. New therapeutics must be developed for aquaculture to avoid overdependence on one specific drug. Development of new efficacious vaccines, both monovalent and bivalent, novel mass immunization methods and broad application of vaccines should be continued for prevention of ES and ESC.

Note

[1]Mention of trade names does not imply recommendation or endorsement by the US Department of Agriculture.

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15 Vibriosis

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Introduction

Vibriosis is one of the most prevalent fish diseases caused by bacteria belonging to the genus Vibrio. Vibriosis caused by *Vibrio anguillarum* has been particularly devastating in the marine culture of salmonid fish. This fish pathogen was first described in 1909 by Bergman as the aetiological agent of 'Red Pest of eels' in the Baltic Sea (Bergman, 1909). Before this report, Canestrini (1893) described epizootics in migrating eels (*Anguilla vulgaris*) dating back to 1817 that implicated a bacterium named *Bacillus anguillarum*. The pathology of the disease and the characteristics of the bacterium in these two reports suggested that the aetiological agents were the same. Vibriosis was not reported in North America until 1953, when *V. anguillarum* was isolated from chum salmon (*Oncorhynchus keta*) (Rucker *et al*., 1953). Outbreaks affecting close to 50 species of fresh- and salt-water fish have been reported in several countries in the Pacific, as well as the Atlantic coasts (Anderson and Conroy, 1970; Strout *et al*., 1978; Tolmasky *et al*., 1985, 1988a). Vibriosis caused by *V. anguillarum* has been recognized as a major obstacle for salmonid marine culture because of the significant losses it causes in the fish industry (Schiewe *et al*., 1977; Winton *et al*., 1983; Trust, 1986; Toranzo *et al*., 2005). More than 30 years ago, Harrell *et al*. (1976) demonstrated that isolates of *V. anguillarum*, the most important aetiological agent of vibriosis, exhibited a marked heterogeneity which led to the division of these vibrios into two separate biotypes, 1 and 2 (Schiewe *et al*., 1977). Later, Schiewe *et al*. (1981) proposed a new species for the *V. anguillarum* biotype 2, based on cultural and biochemical characteristics, as well as DNA homology with biotype 1. This new species was named *V. ordalii* (Schiewe *et al*., 1981).

Other members of the genus *Vibrio* have been isolated in outbreaks of vibriosis in fish and shellfish. These aetiological agents include *V. salmonicida*, a pathogen isolated in the Norwegian coasts causing Hitra disease or Cold Water Vibriosis; *V. damsela* (Love *et al*., 1981); *V. vulnificus* biotype II (Tison *et al*., 1982); *V. tubiashii* (Hada *et al*., 1984); *V. carchariae* (Grimes *et al*., 1984) and *V. cholerae* non-O1 (Muroga *et al*., 1976; Yamanoi *et al*., 1980). *V. splendidus* and *V. pelagius*, atypical *V. anguillarum* strains and strains from the environment, were isolated from infected fish in the Atlantic coast of Spain and Norway (Toranzo and Barja, 1990; Toranzo *et al*., 2005).

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Aetiological Agents of Vibriosis

Vibrio anguillarum

The bacterium

V. anguillarum has suffered many nomenclatural changes from *Beneckea anguilllara* biotype 1 (Baumann *et al.*, 1978) to, more recently, *Listonella anguillarum* (McDowell and Colwell, 1985). However, recent complete genome sequence demonstrated that the original designation was correct (Crosa *et al.*, in preparation), validating the return to the original *V. anguillarum* nomenclature. *V. anguillarum* is a polar flagellated, Gram-negative curved rod (Fig. 15.1) that is a facultative anaerobe with a G + C content of 43–46%. It grows rapidly at 25–30°C in rich media such as brain–heart, trypticase soy broth or agar containing 1.5% NaCl. On solid medium, it produces circular, cream-coloured colonies. *V. anguillarum* belongs to one of the halophilic groups of vibrios and survives at different salinities. Hoff (1989) has shown that it is able to survive in seawater for more than 50 months. A list of the phenotypic properties, as well as the salt and temperature range for *V. anguillarum*, has been published (Schiewe and Crosa, 1981).

The disease

Vibriosis occurs in cultured and in wild marine fish in salt or brackish water, particularly in shallow waters during late summer. It was believed originally that scavenger fish feeding around the farms were the natural reservoir of *V. anguillarum*, and contact between fish seemed to be an important factor for the spread of this pathogen. However, there is evidence that *V. anguillarum* is normally present in the food of cultured and wild healthy fish (Roberts, 1989). The temperature and quality of the water, the virulence of the *V. anguillarum* strain and stress on the fish are important elements influencing the onset of disease outbreaks. The characteristic clinical signs of vibriosis include red spots on the ventral and lateral areas of the fish and swollen and dark skin lesions that ulcerate, releasing a blood exudate (Fig. 15.2). There are also corneal lesions characterized by an initial opacity, followed by ulceration and evulsion of the orbital contents. However, in acute and severe epizootics, the course of the infection is rapid and most of the infected fish die without showing any clinical signs. The reviews by Toranzo and her collaborators (Toranzo and Barja, 1990; Toranzo *et al.*, 2005) detail most of the reports of

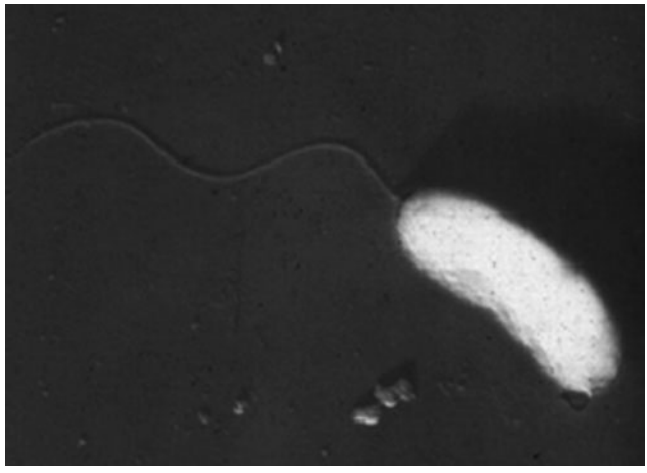


Fig. 15.1. Electron micrograph of *V. anguillarum* showing a single polar flagellum. Shadowed preparation × 10,000 (micrograph by Dr J.H. Crosa).

V. anguillarum-caused vibriosis in cultured fish, molluscs and crustacea. Table 15.1, adapted from these publications, lists the vibriosis outbreaks caused by *V. anguillarum* in different countries.

Ransom *et al.* (1984) studied and compared the histopathology of vibriosis caused by *V. anguillarum* and *V. ordalii* in rainbow trout and salmon and found significant differences between the diseases caused by these bacteria. However, it was suggested, based on DNA homology and serological studies, that *V. ordalii* was just a biotype of *V. anguillarum* (Toranzo *et al.*, 2005). In this section, we will describe vibriosis caused by *V. anguillarum*. This disease is characterized by a haemorrhagic septicaemia. The number of leucocytes is reduced (Ransom *et al.*, 1984) and darkening of the diseased fish is noted with petechiae at the base of fins and skin. Ulcers can also be observed. Cisar and Fryer (1969) reported that the intestine became distended and filled with a clear, viscous liquid. *V. anguillarum* was found in large numbers in the blood and haematopoietic tissues. The pathology is more severe in the descending gastrointestinal tract and rectum than in the anterior region due to a pH gradient, which is alkaline in the rectum and becomes acidic towards the anterior gastrointestinal tract. Ransom *et al.* (1984) demonstrated that *V. anguillarum* did not grow in an acidic medium. Histological examination of infected rainbow trout tissues demonstrated the location of the *V. anguillarum* during infection (Nelson *et al.*, 1985a,b). The bacterium was found initially in the spleen, but as the number of cells in this organ increased, bacteria appeared in the kidney. At the time of death, most tissues were septic and no phagocytosis by macrophages was detected. Severe cardiac myopathy, renal and splenic necrosis and periorbital oedema were also described in preacute vibriosis cases (Roberts, 1989).

Identification and classification

Outbreaks of *V. anguillarum* may lead to a rapid loss of farmed fish. Therefore, a quick diagnosis to minimize fish losses is essential. Identification methods include a culture medium for presumptive identification, a sensitivity assay to filter discs impregnated with a saturated solution of the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylteridine), nitrate reduction, presence of oxidase, catalase and arginine decarboxylase, reaction with monoclonal antibodies and anti-flagellar antiserum and hybridization with specific 16S rRNA oligonucleotides (Shewan *et al.*, 1954; Larsen, 1983; Tassin *et al.*, 1983; Rehnstam *et al.*, 1989; Alsina *et al.*, 1994; Martinez-Picado *et al.*, 1994). Identification of *Vibrio* species that possess similar biochemical and morphological properties can be achieved by using



Fig. 15.2. Juvenile coho salmon (*Oncorhynchus kisutch*) exhibiting some of the common signs of vibriosis. The extensive haemorrhaging along the abdominal surface is typical of the chronic form of this disease (photograph provided by David Bruno).

Table 15.1. Vibriosis outbreaks caused by *Vibrio anguillarum* (from Toranzo and Barja, 1990).

Species	Country
Pacific salmon	
Oncorhynchus kisutch	USA, Japan, Spain
O. keta, O. nerka, O. gorbuscha	Canada
O. masou, O. rhodurus	Japan
O. tshawytscha	USA, Canada
Atlantic salmon	
Salmo salar	Norway
Trout	
O. mykiss	USA, Japan, Italy, Norway, Denmark, Spain
S. trutta	Scotland
Turbot	
Scophthalmus maximus	Scotland, Spain
Striped bass	
Morone saxatilis	USA
Winter flounder	
Pseudopleuronectes americanus	USA
Cod	
Gadus morhua	Norway, Denmark
Red sea bream	
Pagrus major	Japan
European eel	
Anguilla anguilla	Norway
Japanese eel	
A. japonica	Japan
Saithe	
Pollachius virens	Norway
Gilthead sea bream	
Sparus aurata	Israel
Sea mullet	
Mugil cephalus	Scotland
Seriola	
Seriola quinqueradiata	Japan
Channel catfish	
Ictalurus punctatus	USA
Milkfish	
Chanos chanos	Taiwan
Ayu	
Plecoglossus altivelis	Japan
Tilapia	
Oreochromis aureus	Kuwait
European oyster	
Ostrea edulis	USA, UK, Spain
Japanese oyster	
Crassostrea virginica	USA
Clam	
Mercenaria mercenaria	USA
Lobster	
Homarus americanus	USA
Shrimp	
Penaeus sp.	USA

monoclonal antibodies (MAbs) prepared against sodium azide-killed cells (Chen *et al.*, 1992). Three types of MAbs were analysed and enzyme-linked immunosorbent assays (ELISA) and fluorescein isothiocyanate (FITC) immunofluorescence tests showed that the genus-specific MAbs were very useful for identifying vibrios, while the species-specific MAbs were useful for completing the diagnosis. Most of these biochemical and immunological methods require the culture and isolation of the infecting bacteria from the fish. Conversely, newer hybridization techniques do not require a pure culture. Rehnstam *et al.* (1989) and Martinez-Picado *et al.* (1994) described synthetic oligonucleotides used as specific probes for the identification of *V. anguillarum*. These oligonucleotides were designed using the information generated after sequencing the 16S rRNA from several *V. anguillarum* strains. The radiolabelled nucleotides were used as probes in DNA hybridization assays, which were carried out using purified DNA and homogenized fish tissues, such as kidney. The hybridization of these probes against *V. anguillarum* DNA is very specific, with no cross-hybridization against other bacterial species. This molecular assay permits the identification of *V. anguillarum* within 24 h.

A multiplex PCR approach was developed for the detection of haemolysin-producing *V. anguillarum* strains using primers targeting five haemolysin genes (*vah1*, *vah2*, *vah3*, *vah4* and *vah5*) (Rodkhum *et al.*, 2005, 2006a). This method was successful in amplifying reactions containing as little as 100 fg of genomic template DNA. The direct detection of *V. anguillarum* in clinical specimens by this multiplex PCR was also successful in reactions containing as few as 10 bacterial cells. This multiplex PCR method can be a rapid and sensitive method for detecting pathogenic *V. anguillarum*.

Serotypes

In comparative studies, pathogenic, environmental and reference strains are similar, but no cross-reacting antigens are present in these strains (Larsen, 1983). Therefore, the classification of *V. anguillarum* by serological methods has proved to be convenient (Pacha and Kiehn, 1969; Johnsen, 1977; Kitao *et al.*, 1983; Sorensen and Larsen, 1986). A serotyping scheme has been proposed based on the detection by slide agglutination of *V. anguillarum* O antigens (Sorensen and Larsen, 1986). Using this test, Toranzo *et al.* (1987) detected the presence of this pathogen in infected fish. Ten serotypes (O1–O10) have been described in *V. anguillarum*; however, most vibriosis outbreaks involving cultured salmonid fish and feral marine fish have been shown to be caused by strains belonging to serotypes O1 and O2, respectively (Toranzo and Barja, 1990). Strains belonging to serotypes O3–O10 have been isolated mainly from marine environmental samples, including water, sediment, phyto- and zooplankton. Bolinches *et al.* (1990) used a combination of immunological methods, including double immunodiffusion, dot blot assay and an enzyme-linked immunoabsorbent technique, and established that the O2 group could be subdivided into two subgroups, O2a and O2b. This finding agreed with that of Rasmussen (1987a), who demonstrated that two lipopolysaccharide species, nominated O2a and O2b, were present in O2 strains. Bacteria belonging to the O2a group were isolated from salmonids and non-salmonid fish; strains belonging to the O2b group were isolated from non-salmonids only. The question of whether the heterogeneity observed within the O2 also occurs in other serotypes awaits further studies. The presence of capsular antigens (K antigens) has been reported for *V. anguillarum* of the O1, O4, O5 and O6 groups (Rasmussen, 1987b,c; Tajima *et al.*, 1987a,b). However, the role of K antigens in pathogenicity remains to be determined. Agglutination has also been used to type environmental and fish isolates (Larsen and Mellegaard, 1984). All *V. anguillarum* isolates exerted either mannose-sensitive haemagglutination or were non-agglutinating. The *V. anguillarum* agglutinins have specificity against human, poultry, guinea pig and trout erythrocytes, as well as yeast cells. Therefore, a scheme was developed based

on the agglutination ability of the different isolates for specific eukaryotic cells. Eight different agglutination types (A through H) were defined by this method.

The correlation between serotype and pathogenicity may reflect the ability of the bacterial surface antigens to interact with the host tissues. Furthermore, studies of surface components such as outer membrane proteins and lipopolysaccharides of *V. anguillarum* strains demonstrated that these bacterial components were related to the serotypes of the pathogens (Aoki *et al.*, 1981; Nomura and Aoki, 1985).

Analysis of a library of approximately 20,000 transposon-insertion derivatives resulted in the identification of chromosomal genes involved in the synthesis of the siderophore anguibactin, as well as in ferric anguibactin utilization. Genetic and sequence analyses of one such transport-defective mutant revealed that the transposon insertion occurred in an open reading frame (ORF) with homology to *rmlC*, a dTDP-rhamnose biosynthetic gene (Welch and Crosa, 2005). This ORF resides within a cluster of four ORFs, all of which are predicted to function in the biosynthesis of this O side-chain precursor. The same phenotype was seen in a mutant obtained by allelic exchange in *rmlD*, another ORF in this dTDP-rhamnose biosynthetic cluster. This mutation could be complemented with the wild-type *rmlD* gene, restoring both production of the O1 antigen side chain and ferric anguibactin transport. Presence of the O1 side chain was crucial for the resistance of *V. anguillarum* to the bactericidal action of non-immune serum from the fish host. Surprisingly, further analysis demonstrated that these mutations were pleiotropic, leading to a dramatic decrease in the levels of FatA, the outer membrane protein receptor for ferric anguibactin transport, and a concomitant reduction in iron transport. Thus, our results in this work demonstrate that the lipopolysaccharide O1 side chain is required for the operation of two critical virulence factors in *V. anguillarum*: serum resistance and anguibactin-mediated iron transport. These factors allow *V. anguillarum* to survive in serum and multiply in the iron-limiting milieu of the host vertebrate.

Virulence factors

IRON ACQUISITION – THE ANGUIBACTIN SYSTEM. *V. anguillarum* virulence is associated with the presence of a plasmid-mediated iron-uptake system (Crosa, 1980). The 65 kilobase pairs (kb) pJM1 plasmid present in the prototype O1 strain *V. anguillarum* 775 encodes a very efficient iron-sequestering system consisting of the indigenous siderophore anguibactin, as well as transport and regulatory genes (for recent reviews see Crosa and Walsh, 2002; Lopez and Crosa, 2007). The iron uptake system encoded in plasmid pJM1 enables *V. anguillarum* to cause a highly fatal haemorrhagic septicaemic disease in salmonids and other fish, leading to epizootics throughout the world. The biosynthesis genes for the siderophore anguibactin are encoded by both the pJM1 plasmid and the chromosome, while those involved in the transport of the ferric-siderophore complex, including the outer membrane receptor, are plasmid-encoded (Alice *et al.*, 2005). The *in vivo* expression of the pJM1-encoded iron-uptake system was assessed by carrying out experimental co-infections using the 775 wild-type strain and an avirulent insertional mutant, affected in the anguibactin biosynthesis but not in the receptor activity for this siderophore (Wolf and Crosa, 1986). This classic virulence experiment demonstrated that the mutant impaired in the synthesis of anguibactin could be recovered from dead fish only when co-injected with the 775 wild type. This observation also demonstrated that anguibactin is indeed produced and secreted into the bloodstream of the natural host during the process of infection. Once in the bloodstream, anguibactin can scavenge iron from the host and deliver this essential metal to the bacterial cytosol. These experiments not only show that anguibactin is produced *in vivo*, but also that it is an important factor of virulence. This was the first direct demonstration of the involvement of a siderophore in bacterial virulence.

Complete sequence of the pJM1 plasmid 65,009-nucleotide sequence, with an overall G + C content of 42.6%, revealed genes and ORFs encoding iron transporters, non-ribosomal peptide enzymes and other proteins essential for the biosynthesis of the siderophore anguibactin (Fig. 15.3) (Di Lorenzo *et al.*, 2003). Of the 59 ORFs, approximately 32% were related to iron metabolic functions. The *fatDCBA-angRT* operon, as well as other downstream biosynthetic genes, are bracketed by the homologous ISV-A1 and ISV-A2 insertion sequences. Other gene clusters on the plasmid also show an insertion element-flanked organization, including ORFs homologous to genes involved in the biosynthesis of 2,3-dihydroxybenzoic acid. Homologues of replication and partition genes are also identified on pJM1 adjacent to this region. ORFs with no known function represent approximately 30% of the pJM1 sequence. The insertion sequence elements in the composite transposon-like structures, corroborated by the G + C content of the pJM1 sequence, suggest a modular composition of plasmid pJM1, biased towards acquisition of modules containing genes related to iron metabolic functions. This work also shows that there is considerable

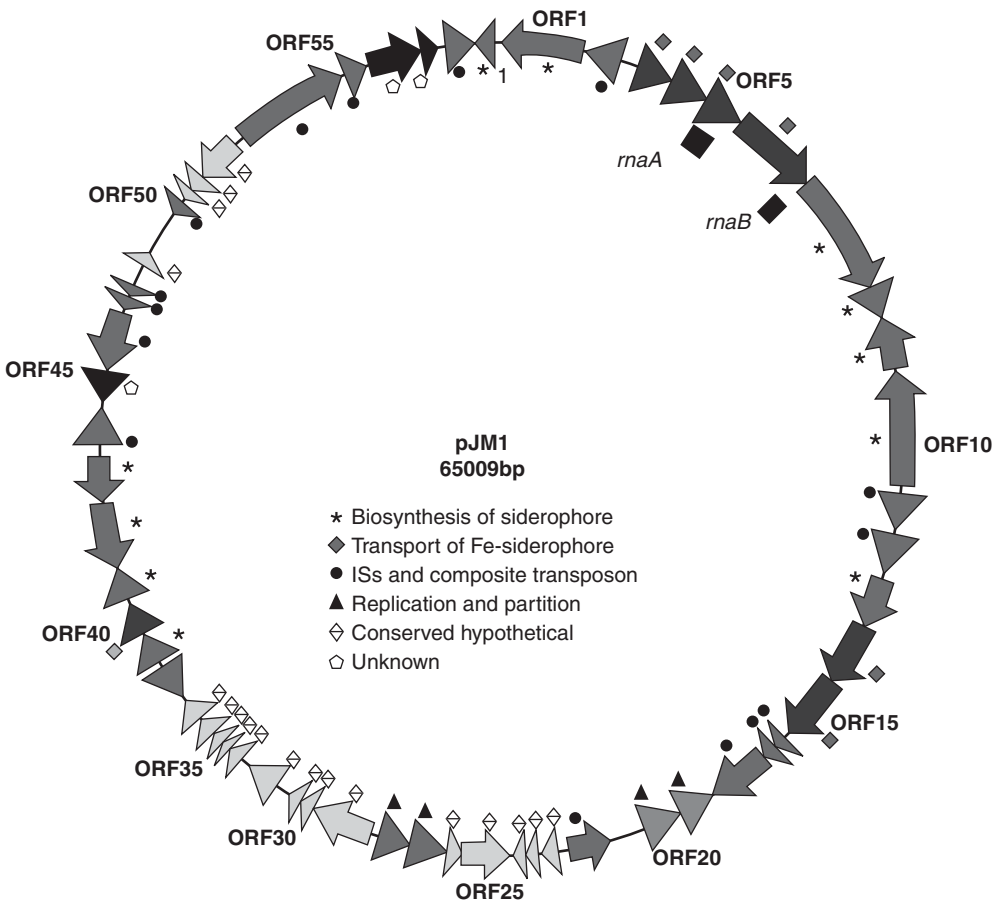


Fig. 15.3. Genetic map of the *V. anguillarum* 775 pJM1 plasmid. Schematic representation of the ORFs on the two strands of pJM1 DNA. The black blocks represent genes encoding antisense RNAs: *rnaA,* antisense RNAα, and *rnaB,* antisense RNAβ (adapted from Di Lorenzo *et al.*, 2003).

microheterogeneity in pJM1-like plasmids from virulent strains of *V. anguillarum* isolated from different geographical sources, although conserving the greater homology picture.

The pJM1 plasmid-mediated system is expressed maximally under iron-limiting conditions and requires the bifunctional protein AngR that acts as a positive regulator of anguibactin biosynthesis and also possesses an enzymatic function that may play a role in anguibactin biosynthesis (Wertheimer *et al.*, 1999). AngR also positively regulates transcription of the iron-transport genes *fatA* and *fatB*, and transcription of *angR* is repressed by Fur under iron-rich conditions. In addition, anguibactin itself enhances transcription of the iron-transport genes *fatA* and *fatB*, independently of AngR and the *trans*-acting factor (TAF) product(s). The presence of either AngR (together with the TAF product(s)) or anguibactin alone leads to a partial level of expression of the iron-transport genes *fatA* and *fatB*, while full expression is achieved when AngR, the TAF products and anguibactin are all present (Wertheimer *et al.*, 1999).

ANGUIBACTIN BIOSYNTHESIS. The siderophore anguibactin has been purified from the supernatant of *V. anguillarum* 775 cultures grown under iron starvation (Actis *et al.*, 1986) and characterized as w-*N*-hydroxy-w-*N*[[2'-(2",3"-dihydroxyphenyl)thiazolin-4'-yl]carboxy]histamine (Fig. 15.4) (Jalal *et al.*, 1989). Although most siderophores can be classified in two main groups, catechols and hydroxamates (Neilands, 1983), anguibactin belongs to a unique structural class of its own, harbouring a catechol moiety together with a hydroxamate residue. The stoichiometry of anguibactin to ligand

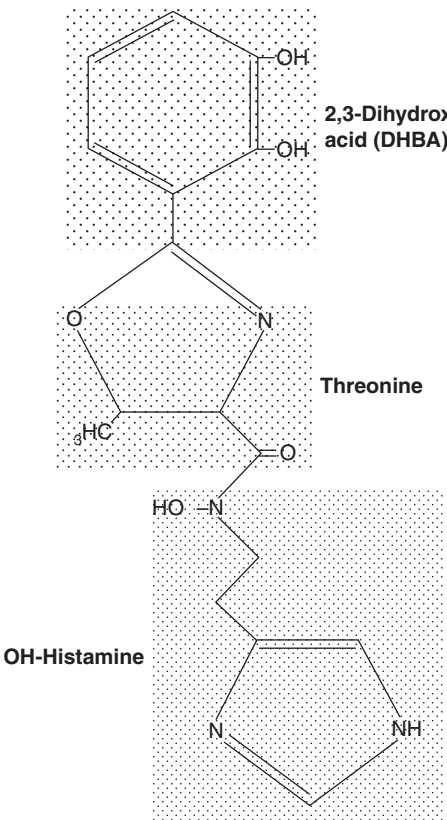


Fig. 15.4. Structure of anguibactin. The ferric iron is coordinated by the *O*-hydroxy group, the nitrogen of the thiazoline ring, the hydroxamate and the deprotonated nitrogen of the imidazole ring (Jalal *et al.*, 1989).

was determined *in vitro* using Ga(III) and it was shown to be 1:1 (Jalal *et al.*, 1989).

The *angB/G*, *angM*, *angN*, *angR* and *angT* genes, which code for non-ribosomal peptide synthetases (NRPSs), catalyse the synthesis of anguibactin (Di Lorenzo *et al.*, 2003, 2004, 2008). In addition, the genes *fatA*, *fatB*, *fatC* and *fatD* are involved in the transport of ferric-anguibactin complexes. These transport genes, together with the biosynthesis genes *angR* and *angT*, are included in the iron-transport biosynthesis (ITB) operon. Both the biosynthesis and the transport genes are under tight positive as well as negative control. A chromosomally encoded Fur and a plasmid-mediated antisense RNA, RNA beta, act as negative regulators, while positive regulation is facilitated by AngR and TAFr (Wertheimer *et al.*, 1999; Stork *et al.*, 2007a). In addition, the siderophore itself plays a positive role in the regulatory mechanism of the expression of both transport and biosynthesis genes. Products encoded in the TAF region are necessary for the biosynthesis of anguibactin and for maximal expression of iron transport and biosynthesis genes in the plasmid-encoded iron-scavenging system of *V. anguillarum*. Our results also show that the regulatory functions of TAF are encoded in a region, TAFr, which is distinct from and independent of the *angB* and *angG* genes.

Anguibactin, the siderophore produced by *V. anguillarum* 775, is synthesized via an NRPS mechanism. Most of the genes required for anguibactin biosynthesis are harboured by the pJM1 plasmid. Complete sequencing of this plasmid identified an *orf* encoding a 108 kDa predicted protein, AngN. AngN is essential for anguibactin biosynthesis and possesses two domains with homology to cyclization (Cy) domains of NRPSs. Substitution by alanine of the aspartic acid residues within a conserved motif of either the Cy1 or Cy2 domains demonstrated the importance of these two domains in AngN function during siderophore biosynthesis. Site-directed mutations in both domains (D133A/D575A and D138A/D580A) resulted in anguibactin-deficient phenotypes, while mutations in each domain did not abolish siderophore production but caused a reduction in the amounts produced (Di Lorenzo *et al.*, 2008). The mutations D133A/D575A and D138A/D580A also resulted, as expected, in a dramatic attenuation of the virulence of *V. anguillarum* 775, highlighting the importance of this gene for the biosynthesis of anguibactin within the vertebrate host. Regulation of the *angN* gene follows the patterns observed at the iron transport–biosynthesis promoter, with *angN* transcription repressed in the presence of iron and enhanced by AngR and *trans*-acting factor (TAF) under iron limitation.

FERRIC ANGUIBACTIN TRANSPORT. Anguibactin is synthesized in the cytoplasm and then excreted outside the cell, where it competes for iron with the high-affinity iron-binding proteins, such as transferrin, present in the fish blood. The Fe(III)-anguibactin complex is recognized specifically by the membrane receptor and internalized into the cell cytosol. The membrane transport components involved in this process included at least four proteins, FatA, FatB, FatC and FatD, which were shown to be encoded by the pJM1 plasmid (Actis *et al.*, 1988; Köster *et al.*, 1991) (Fig. 15.5). Sequence analysis of the pJM1 region encoding these proteins showed topological homologies between these transport components and those described in *Escherichia coli* for the transport of Fe(III)-citrate, Fe(III)-hydroxamates and -catecholates, and vitamin B_{12} (Köster *et al.*, 1991). Of the four components, the best characterized is the 86 kDa FatA protein, which is only detected when cells are grown under iron-limiting conditions (Crosa and Hodges, 1981). This protein is located on the outer membrane and is exposed to the extracellular environment (Actis *et al.*, 1985). Analysis of the FatA sequence also revealed the presence of a TonB box in FatA. The TonB protein is presumably responsible for energy coupling to the outer membrane (Heller *et al.*, 1988; Pressler *et al.*, 1988). FatB is the putative anguibactin-binding protein. It is a lipoprotein that is anchored in the inner membrane facing the periplasmic space (Actis *et al.*, 1995), while FatD and FatC are integral membrane

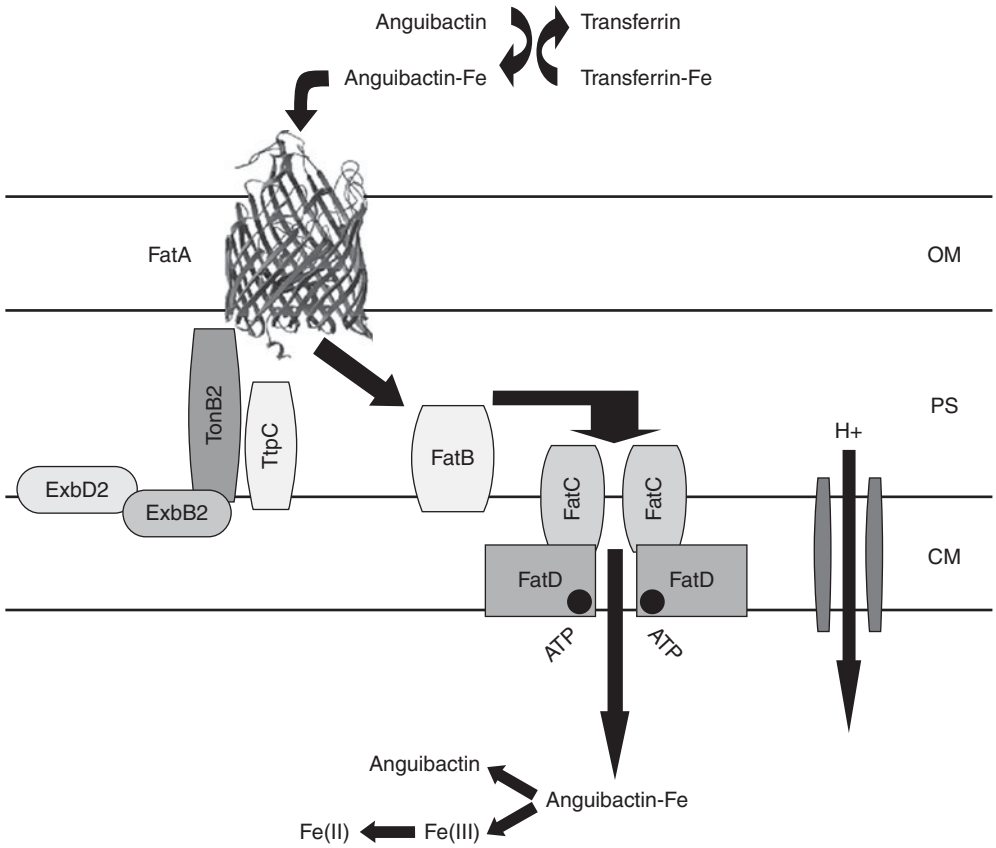


Fig. 15.5. Model of the ferric anguibactin transport system. The FatABCD transport system is coded by genes located in the pJM1 plasmid of *V. anguillarum* 775. The FatA outer membrane (OM) protein is the receptor that recognizes the ferric anguibactin complexes, which are translocated into the periplasmic space (PS) in an energy-dependent process supplied by the ExbB2-ExbD2-TonB2-TtpC energy transducing system. The ferric anguibactin complexes are captured by the periplasmic binding liprotein FatB, which delivers these complexes to the permease system composed of FatC and FatD located in the cytoplasmic membrane (CM). Once in the cytoplasmic space, iron is liberated from the ferric anguibactin complexes and used for a wide range of physiological processes.

proteins (Köster *et al.*, 1991). Based on their homologies to other iron-transport systems, these last two proteins appear to be involved in the translocation of the Fe(III)-anguibactin complexes across the cytoplasmic membrane (Köster *et al.*, 1991).

We recently described the role of specific amino acid residues of the outer membrane receptor FatA in the mechanism of transport of ferric-anguibactin. FatA modelling indicated that this protein had a 22 stranded beta-barrel blocked by the plug domain, the latter being formed by residues 51–154. Deletion of the plug domain resulted in a receptor unable to act as an open channel for the transport of the ferric-anguibactin complex (Lopez *et al.*, 2007).

Active transport across the outer membrane in Gram-negative bacteria requires the energy that is generated by the proton motive force in the inner membrane. This energy is transducer to the outer membrane by the TonB protein in complex with the proteins ExbB and ExbD. *V. anguillarum* harbours two TonB systems, TonB1 and TonB2; the latter is used for

ferric-anguibactin transport and is transcribed as part of an operon that consists of *orf2*, *exbB2*, *exbD2* and *tonB2* (Stork *et al.*, 2004, 2007b).

This gene cluster was identified by a polar transposon insertion in *orf2* that resulted in a strain deficient for ferric-anguibactin transport. Only the entire cluster (*orf2*, *exbB2*, *exbD2* and *tonB2*) could complement for ferric-anguibactin transport, while just the *exbB2*, *exbD2* and *tonB2* genes were unable to restore transport. This suggests an essential role for this Orf2, designated TtpC, in TonB2-mediated transport in *V. anguillarum*. A similar gene cluster exists in *V. cholerae*, i.e. with the homologues of *ttpC-exbB2-exbD2-tonB2*, and we demonstrate that TtpC from *V. cholerae* also plays a role in the TonB2-mediated transport of enterobactin in this human pathogen. Furthermore, the *V. anguillarum* TtpC protein is found as part of a complex that might also contain the TonB2, ExbB2 and ExbD2 proteins. This novel component of the TonB2 system found in *V. anguillarum* and *V. cholerae* is perhaps a general feature in bacteria harbouring the *Vibrio*-like TonB2 system (Stork *et al.*, 2004, 2007b).

REGULATION

Fur Expression of the iron transport genes *fatDCBA* in *V. anguillarum* 775 is regulated negatively by two iron-responsive repressors, the Fur protein and the antisense RNA, RNA alpha. The iron transport promoter was localized in a region approximately 300 base pairs upstream of *fatD*. High activity of the promoter was measured in response to iron depletion in the wild-type strain when a promoter–*lacZ* fusion was examined, whereas the promoter was constitutive in the Fur-deficient strain. Fur binds specifically to two contiguous sites comprising the promoter region and the region downstream of the transcription start site. The identified Fur binding sites showed a low degree of homology to each other as well as to the consensus sequence for the *E. coli* Fur protein. DNase I footprint patterns suggested a sequential interaction of Fur with these two sites that rendered a protection in the template strand and a hypersensitivity to the nuclease in the non-template strand. The periodicity of the hypersensitive sites suggested that the promoter DNA underwent a structural change on binding to Fur, which might play a role in the repression of gene expression (Chai *et al.*, 1998).

AngR AngR, a 110 kDa protein component of this system, appears to play a role in both regulation of the expression of the iron transport genes *fatDCBA* and the production of the siderophore anguibactin. Genetic and molecular evidence from transposition mutagenesis experiments and RNA analysis indicates that *angR*, which maps immediately downstream of the *fatA* gene, is part of a polycistronic transcript that also includes the iron transport genes *fatDCBA* and *angT*, a gene located downstream of *angR* which shows domain homology to certain thioesterases involved in non-ribosomal peptide synthesis of siderophores and antibiotics. The evidence, while not rigorously eliminating the possibility that a separate regulatory polypeptide exists and is encoded somewhere within the 5'-end region of the *angR* gene, strongly supports the idea that AngR is a bifunctional protein and that it plays an essential role in the virulence mechanisms of *V. anguillarum*.

Many *V. anguillarum* strains isolated from various geographical regions carry plasmids highly related, albeit not identical, to pJM1 (Tolmasky *et al.*, 1985, 1988b). Some of those pJM1-like plasmids, chiefly those present in strains isolated from the Atlantic ocean, specified an increased anguibactin production as compared to the pJM1-mediated anguibactin levels found with *V. anguillarum* strains isolated from the Pacific ocean (Tolmasky *et al.*, 1988b). The *angR* gene of one of these pJM1-like plasmids was identified as the responsible element for the enhanced biosynthesis of anguibactin in the strain 531A (Tolmasky *et al.*, 1988b, 1993; Salinas *et al.*, 1989). Polymorphisms in pJM1-like plasmids were also observed in several other *V. anguillarum*

strains belonging to serotype O1 (Wiik *et al.*, 1989b; Olsen and Larsen, 1990).

RNA beta The iron transport–biosynthesis operon in *V. anguillarum* includes four genes for ferric siderophore transport, *fatD*, *-C*, *-B* and *-A*, and two genes for siderophore biosynthesis, *angR* and *angT*. Despite being part of the same polycistronic mRNA, the relative levels of transcription for the *fat* portion and for the whole ITB message differ profoundly, the levels of the *fat* transcript being about 17-fold higher. Using S1 nuclease mapping, *lacZ* transcriptional fusions and *in vitro* studies, we were able to show that the differential gene expression within the ITB operon was due to termination of transcription between the *fatA* and *angR* genes, although a few transcripts proceeded beyond the termination site to the end of this operon. This termination process requires a 427-nucleotide antisense RNA, RNA beta, that spans the intergenic region and acts as a novel transcriptional terminator (Stork *et al.*, 2007a).

IRON ACQUISITION – THE VANCHROBACTIN SYSTEM. A novel chromosome cluster of genes in *V. anguillarum* 775 that includes redundant functional homologues of the pJM1 plasmid-harboured genes, *angE* and *angC*, which are involved in anguibactin biosynthesis, has been identified recently (Alice *et al.*, 2005). A chromosomal *angA* gene that is essential in anguibactin biosynthesis is also included in this gene cluster (Alice *et al.*, 2005). This chromosomal gene cluster encoding vanchrobactin biosynthesis and transport genes was identified in the *V. anguillaru*m serotype O1 strain, 775(pJM1), harbouring the anguibactin biosynthetic genes in the pJM1 plasmid. In this strain, only anguibactin is produced, as the vanchrobactin chromosome cluster has an RS1 transposition insertion into *vabF*, one of the vanchrobactin biosynthesis genes. Removal of this RS1 generating 775(pJM1) Delta *tnp* still resulted in the detection of only anguibactin in specific bioassays. Surprisingly, when the pJM1 plasmid was not present, as in the plasmidless strain H775-3, removal of the RS1 resulted in the detection of vanchrobactin only. As HPLC and mass spectrometry analysis demonstrated that both vanchrobactin and anguibactin were indeed produced in 775(pJM1) Delta *tnp*, it was clear that the pJM1-encoded anguibactin siderophore had higher affinity for iron than the vanchrobactin system in strains in which both systems were expressed at the same time. Our results underscore the importance of the anguibactin system in the survival of *V. anguillarum* 775 under conditions of iron limitation (Naka *et al.*, 2008).

Other virulence factors

HAEMOLYSINS. Dr Crosa's laboratory, together with his collaborators (Rodkhum *et al.*, 2005), identified four chromosomal nucleotide sequences showing homology to known haemolysin genes, which were cloned and sequenced from *V. anguillarum* strain H775-3. The four genes, *vah2*, *vah3*, *vah4* and *vah5*, have ORFs encoding polypeptides of 291, 690, 200 and 585 amino acid residues, respectively, with predicted molecular masses of 33, 75, 22 and 66 kDa, respectively. VAH2 is related most closely to a putative haemolysin of *V. vulnificus* YJ016 (89% identity). VAH3 is related most closely to a protein in *V. cholerae* O1 (68% identity). VAH4 is related most closely to a thermostable haemolysin in *V. cholerae* O1 (72% identity) and VAH5 is related most closely to a putative haemolysin in *V. cholerae* O1 (73% identity). The purified haemolysin proteins showed haemolytic activities against fish, sheep and rabbit erythrocytes. Four strains of *V. anguillarum* mutants were constructed, each deficient in one of the haemolysin genes. Each mutant was less virulent than *V. anguillarum* H775-3 to juvenile rainbow trout (*O. mykiss*), indicating that each haemolysin gene contributed to the virulence of *V. anguillarum* H775-3. This laboratory also identified a homologue of the *V. cholerae* RTX toxin gene in *V. vulnificus*, which corresponded to the coding sequence VV20479 and was therefore named, *rtxA1* (Liu *et al.*, 2007). This gene, which is part of a polycistronic operon whose expression is controlled

by the HlyU transcriptional regulator, proved to be an important factor that determined cytotoxic activity as well as the virulence of this bacterial pathogen.

Recently, a putative *rtx* operon with six genes (*rtxACHBDE*) has also been identified in *V. anguillarum* (Li *et al.*, 2008), where *rtxA* encodes an exotoxin, *rtxC* encodes an RtxA activator, *rtxH* encodes a conserved hypothetical protein and *rtxBDE* encodes the ABC transporters. Strains containing a mutation in *vah1* and a mutation in an *rtx* gene result in a haemolysin-negative mutant, demonstrating that the *rtx* operon is a second haemolysin gene cluster in *V. anguillarum*. It was shown some time ago that *rtxA* was an important virulence factor in *V. vulnificus*, and recently this was also described for the *rtxA* gene of *V. anguillarum* (Li *et al.*, 2008).

HAEMIN AND HAEMOGLOBIN UPTAKE. *V. anguillarum* can utilize haemin and haemoglobin as iron sources. Nine genes, *huvA*, *huvZ*, *huvX*, *tonB1*, *exbB1*, *exbD1*, *huvB*, *huvC* and *huvD*, encoding the proteins involved in haemin transport and utilization, are clustered in a 10 kb region of chromosomal DNA (Mourino *et al.*, 2004, 2006). Reverse transcriptase-PCR analysis demonstrated that the gene cluster was arranged into three transcriptional units: (i) *huvA*; (ii) *huvXZ*; and (iii) *tonB1exbB1D1-huvBCD*. Transcriptional start sites for each *huvA*, *huvX* and *tonB1* transcript were identified by primer extension analysis, and their respective predicted –10 and –35 promoter regions showed significant similarity to those of sigma 70 recognized promoters. Expression from the three promoters, as analysed by transcriptional fusions to a promoterless *lacZ* gene, was regulated by the iron concentration. Furthermore, analysis of the beta-galactosidase activities of these fusions in a *V. anguillarum fur* mutant demonstrated that the repressor protein Fur was directly involved in the negative iron-mediated regulation of the haemin uptake cluster (Mourino *et al.*, 2006).

RESISTANCE TO THE BACTERICIDAL ACTION OF FISH NON-IMMUNE SERUM. It has been demonstrated that fish sera can kill strains of *V. anguillarum*. Thermal tolerance and treatment with either EDTA or EGTA demonstrate that the mechanism for bacterial killing by rainbow trout serum is the alternative complement pathway (Trust *et al.*, 1981). Resistance to this bactericidal action of normal non-immune serum seems to contribute to the virulence of pathogenic *V. anguillarum* (Trust *et al.*, 1981; Toranzo *et al.*, 1983). Pathogenic strains of *V. anguillarum* isolated from striped bass (*Morone saxatilis*) were resistant to the lytic activity of unheated fish serum, whereas other non-pathogenic *V. anguillarum* strains were sensitive, indicating that this factor might play an important role in the virulence properties of this bacterium (Toranzo *et al.*, 1983). Strains in which the pJM1 plasmid was cured were still resistant to the bactericidal action of the serum, demonstrating that this trait was encoded by the *V. anguillarum* 775 chromosome rather than the pJM1 plasmid (Trust *et al.*, 1981).

PROTEASES AND HAEMAGGLUTINATING ACTIVITY. The production of a protease, another potential virulence factor, has also been described (Norqvist *et al.*, 1990; Farrell and Crosa, 1991). An elastolytic metalloprotease of 36 kDa, requiring Zn^{+2} for its activity and Ca^{+2} for its stability, was found to be associated with the invasion properties of the *V. anguillarum* NB10 strain. Virulence studies using proteolytic activity mutants showed that they had a 1000-fold higher LD_{50} when assayed by immersion and ten-fold higher LD_{50} when assayed by intraperitoneal injection (Norqvist *et al.*, 1990). In addition, Farrell and Crosa (1991) purified a metalloprotease from another *V. anguillarum* strain, 514, that seemed to be either related or identical to that described in strain NB10. The molecular mass of this purified protease was calculated to be 38 kDa. In this case, the protease activity was EDTA sensitive and it could be restored by the addition of both Ca^{+2} and Zn^{+2}. Biochemical analysis, as well as determination of the N-terminal amino acid sequence, showed that the *V. anguillarum* protease(s) were highly related to proteases already

described in other Vibrio species such as *V. cholerae* and *V. vulnificus*, as well as to the elastase of *Pseudomonas aeruginosa* and the protease of *Legionella pneumophila* (Norqvist *et al.*, 1990; Farrell and Crosa, 1991).

Haemagglutination is another bacterial factor that is believed to play a central role in the infectivity of bacterial pathogens. It was found that pathogenic *V. anguillarum* strains isolated from the east coast of the USA produced strong haemagglutinins for fish erythrocytes, while this activity was not detected in non-virulent strains (Toranzo *et al.*, 1983). The pathogenic strains showed two agglutination patterns, depending on the type of erythrocytes agglutinated. This agglutination activity was inhibited by D-mannose, indicating that this carbohydrate or an analogue was part of the erythrocyte receptor for this haemagglutinin. Although a haemagglutinating activity was also detected in *V. anguillarum* strains isolated from the Pacific North-west, no correlation between the expression of this activity and the strain virulence could be established (Trust *et al.*, 1981).

TOXINS. The secretion of a *V. anguillarum* extracellular toxin was reported by Kodama *et al.* (1985). This toxin was purified by gel filtration chromatography, and molecular characterization of the active fraction revealed that the toxic activity was associated with two 44 kDa proteins and a single 34 kDa protein. These proteins were lethal to rainbow trout and mice when injected intraperitoneally and intravenously, respectively. This toxic activity affected the intestinal tract and the vascular system of the injected animals and could be neutralized by a specific antiserum raised in rabbit. Chemical analysis of these components indicated that some of them were associated with carbohydrates. The toxic activity was sensitive to heating and potassium periodate, and resistant to trypsin and acetone treatments. Furthermore, the toxin was not related to either haemolytic or protease activities. This exocellular toxin was another important virulence factor in vibrios since its haemorrhagic, cytotoxic and lethal effects could lead to the pathological alterations observed in infected fish. In another study, Aoki *et al.* (1985) found that a *V. anguillarum* strain became more virulent after passages in ayu (*Plecoglossus altivelis*) and the increase of virulence was correlated with an increase in the amount of production of its lipopolysaccharide. Furthermore, the increase in virulence was also correlated with a change in the size of the lipopolysaccharide; the virulent strain had a high molecular weight component that was absent in the low virulent strain. Exopolysaccharides were also shown to play a role in the virulence of *V. anguillarum* (Croxatto *et al.*, 2007). Mutations in genes involved in exopolysaccharide synthesis and transport, but not lipopolysaccharides, affected the production of exoprotease activity, particularly that due to EmpA, mucinase activity and the ability of the isogenic mutants to attach to and form biofilm on the fish integuments.

MOTILITY AND VIRULENCE. It has been suggested that there is a correlation of the virulence of *V. anguillarum* and the presence of more than one flagellum (Chart, 1983; Norqvist and Wolf-Watz, 1993). Milton *et al.* (1996) cloned and characterized a *V. anguillarum* flagellin gene (*flaA*). Mutations in this gene resulted in partially motile bacteria. Virulence studies concluded that the product of the *flaA* gene was needed for crossing the fish integument and might play a role in virulence (Milton *et al.*, 1996).

Transposition mutagenesis revealed the presence of two genes, *virA* and *virB*, encoding a major surface antigen important for the virulence of *V. anguillarum* (Norqvist and Wolf-Watz, 1993). This surface antigen appears to be a lipopolysaccharide located on the outer sheath of the flagellum and is expressed *in vivo* during fish infections, together with the flagellum. Milton *et al.* (1995) has identified *virC*, another gene that is essential for virulence in *V. anguillarum*. Although mutations in this gene result in strains that lose virulence, the function of the product of *virC* is still unknown.

Many bacterial cells communicate via a process termed quorum sensing. In marine

Vibrio species, the *Vibrio harveyi*-type LuxR protein is a key player in a quorum-sensing phosphorelay cascade, which controls the expression of virulence, symbiotic and survival genes. Milton *et al.* (1997, 2001) have described *V. anguillarum* homologues of LuxR (VanT) and LuxMN (VanMN), LuxPQ (VanPQ) and LuxOU (VanOU). In contrast to other Vibrio species, *vanT* was expressed at low cell density and showed no significant induction as the cell number increased. In addition, although the loss of VanO increased *vanT* expression, the loss of VanU, unexpectedly, decreased it. The signal relay results in a cellular response, as expression of the metalloprotease gene *empA* was altered in a fashion similar to that of *vanT* in all mutants. Consequently, the *V. anguillarum* quorum-sensing phosphorelay systems work differently from those of *V. harveyi* and may be used to limit rather than induce *vanT* expression. However, no significant effect of quorum sensing on virulence was reported. On the other hand, it was found that VanT controlled the expression of genes coding for the metalloprotease EmpA, the first step in the serine–glycine biosynthesis pathway, the production of pigment, as well as the formation of biofilms (Croxatto *et al.*, 2002). Biofilm formation, together with the production of OmpU and resistance to bile, are also under the control of a ToxR homologue, although its mutation results in a slight decrease of virulence (Wang *et al.*, 2002, 2003). Nevertheless, it is interesting to note that some of these regulated *V. anguillarum* products are known as important virulence factors expressed by relevant bacterial pathogens.

PUTATIVE VIRULENCE GENES. Using a total of 2300 clones, 2100 from a plasmid library and 200 from a cosmid library, we obtained partial sequences from the genome of *V. anguillarum* strain H775 that were subjected to homology searches with the BLAST algorithm (Rodkhum *et al.*, 2006b). The total length of the sequenced clones is 1.5 Mbp. The nucleotide sequences were classified into 17 broad functional categories. Forty putative virulence-related genes were identified, 36 of which were novel in *V. anguillarum*, including a repeat in toxin gene cluster, haemolysin genes, an enterobactin utilization gene, protease genes, lipopolysaccharide biosynthesis genes, a capsule biosynthesis gene, flagellar genes and pilus genes.

PHYLOGENETICS. The human pathogen *Acinetobacter baumannii* ATCC 19606^T strain expresses the acinetobactin-mediated iron acquisition system, which is highly related to the iron chelator anguibactin-mediated system expressed by the fish pathogen *V. anguillarum* 775 (Yamamoto *et al.*, 1994; Dorsey *et al.*, 2004; Mihara *et al.*, 2004). Insertional mutagenesis resulted in the isolation of several derivatives whose ability to grow in medium containing the iron chelator 2,2'-dipyridyl was affected. One of the insertions disrupted a gene encoding a predicted outer-membrane protein, named BauA, highly similar to FatA, the receptor for ferric anguibactin. Immunological relatedness of BauA with FatA was confirmed by western blot analysis. Another transposon insertion was mapped to a gene encoding a protein highly similar to FatD, the permease component of the anguibactin transport system. Further DNA sequencing and nucleotide sequence analysis revealed that these *A. baumannii* ATCC 19606^T genes were part of a polycistronic locus that contained the *bauDCEBA* ORFs. While the translation products of *bauD*, *-C*, *-B* and *-A* are highly related to the *V. anguillarum* FatDCBA iron-transport proteins, the product of *bauE* is related to the ATPase component of Gram-positive ATP-binding cassette (ABC) transport systems. This entire locus is flanked by genes encoding predicted proteins related to AngU and AngN, *V. anguillarum* proteins required for the biosynthesis of anguibactin. These protein similarities, as well as the structural similarity of anguibactin and acinetobactin, suggested that these two siderophores could be utilized by both bacterial strains, a possibility that was confirmed by siderophore utilization bioassays (Dorsey *et al.*, 2004). Taken

together, these results demonstrate that these pathogens, which cause serious infections in unrelated hosts, express very similar siderophore-mediated iron-acquisition systems, in spite of the fact that they target different vertebrate hosts in different ecological niches.

Vibrio ordalii

The bacterium

V. ordalii is, together with *V. anguillarum*, *V. salmonicida* and *V. vulnificus* biotype 2, an important causative agent of vibriosis in marine aquaculture. Although it was described initially as the aetiological agent of vibriosis in wild and cultured marine salmonids in the Pacific north-west of the USA, Japan and Australia, it has been found more recently in Atlantic salmon cultured in Chile (Toranzo *et al.*, 2005). This Gram-negative, curved rod with a polar flagellum has been reported using different names such as *Vibrio* sp. 1669 (Harrell *et al.*, 1976), *Vibrio* sp. RT (Ohnishi and Muroga, 1976), *V. anguillarum* biotype 2 (Schiewe *et al.*, 1977) and *B. anguillara* biotype II (Baumann *et al.*, 1978), which was later amended to *V. anguillarum* biotype II and *V. anguillarum* phenon II, until its taxonomical status was clarified by Schiewe *et al.* (1981). Total DNA analysis of a number of strains proved that the mol % G + C content of this fish pathogen DNA was 43–44% and demonstrated that isolates of *V. ordalii* had more than 83% homology within the group, while the homology to *V. anguillarum* DNA was only 58–69% (Schiewe *et al.*, 1981). Furthermore, there is little or no homology with the DNA of other unclassified vibrios as well as with DNA from *V. parahaemolyticus* and *V. alginolyticus*, the latter of which has been associated with epizootics in cultured large yellow croaker (*Pseudosciaena crocea*) caused by infection via the intestinal tract and adhesion to mucus, a process mediated by bacterial surface appendages (Chen *et al.*, 2008). *V. ordalii* was reclassified as *Listonella ordalii* due to the similarity of its 5S rRNA nucleotide sequence to that of other members of the *Listonella* family (Pillidge and Colwell, 1988). More current taxonomical approaches, based on ribotyping and restriction analysis, resulted in the overall reclassification of fish-pathogenic vibrios. These approaches showed that *V. ordalii* strains isolated in North America shared restriction profiles with Australian and Japanese isolates, although each of the geographical groups depicted its own distinct profile (Pedersen *et al.*, 1996). Comparative 16S rRNA sequence analysis (Wiik *et al.*, 1995) also proved to be a convenient tool for the classification of vibrios pathogenic to fish. In general, the evolutionary relationships described with this approach and the previous taxonomy of this bacterial family were in agreement. However, the 16S rRNA sequence analysis is considered necessary for the identification of phenotypically closely related isolates to avoid misidentifications (Montes *et al.*, 2003).

V. ordalii has been isolated from natural and experimental infections in coho salmon (*O. kisutch*) and chum and spring Chinook salmon (*O. tshawytscha*) (Ransom *et al.*, 1984), respectively. This pathogen grows at 30°C in trypticase soy broth or brain–heart infusion in the presence of 1% NaCl. The biochemical characteristics of *V. ordalii* have been described before (Schiewe *et al.*, 1981) and Table 15.2 lists the biochemical tests frequently used to distinguish between *V. ordalii* and *V. anguillarum*. Antigenic differences in their lipopolysaccharides, which can be detected using specific monoclonal or polyclonal antibodies used as diagnostic and serotyping reagents (Mutharia *et al.*, 1993; Mutharia and Amor, 1994), distinguish these two important fish pathogens. The structure of the lipopolysaccharide O-antigen and the capsular polysaccharide of *V. ordalii* explains the antigenic similarities as well as the differences between this pathogen and *V. anguillarum* (Sadovskaya *et al.*, 1998).

The presence of a 30 kb cryptic extrachromosomal element is a common feature among all *V. ordalii* strains examined (Schiewe and Crosa, 1981). This plasmid, known as pMJ101, is unrelated to the

Table 15.2. Phenotypic properties used to differentiate *V. anguillarum* from *V. ordalii* (data taken from Schiewe *et al.*, 1981).

Biochemical tests and growth temperature	*V. anguillarum*	*V. ordalii*
Arginine–alkaline reaction	+	–
Citrate, Christensen	+	–
Citrate, Simmons	+	–
Lipase	+	–
ONPG	+	–
Starch hydrolysis	+	–
Voges–Proskauer	+	–
Acid production from		
Cellobiose	+	–
Glycerol	+	–
Sorbitol	+	–
Trehalose	+	–
Growth at 37°C	+	–

ONPG, *o*-nitrophenyl β-*D*-galactopyranoside.

V. anguillarum 775 pJM1 virulence plasmid and seems to be different from plasmids belonging to known incompatibility groups. These observations were confirmed by later studies (Tiainen *et al.*, 1995; Pedersen *et al.*, 1996), one of which showed that, although highly conserved, the pMJ101-like plasmids isolated from different strains had variations in their restriction patterns (Pedersen *et al.*, 1996). These plasmid variations, together with ribotyping, which showed three *V. ordalii* ribotypes clearly different from those of *V. anguillarum*, could be useful in epidemiological studies of *V. ordalii* (Pedersen *et al.*, 1996).

Classical restriction analysis and cloning and transformation studies in combination with transposition mutagenesis localized the pMJ101 replication functions within a 2.4 kb *Eco*RV-*Hin*dIII restriction fragment (Bidinost *et al.*, 1994). Recombinant derivatives harbouring this fragment replicated in *E. coli* cells deficient in DNA polymerase I (PolI) or integration host factors (IHF). However, the replication of plasmid derivatives containing the pMJ101 *ori* region depended on dam activity, suggesting that the pMJ101 replication depended on active DNA methylation. The stable replication of pMJ101 derivatives in the absence of PolI suggests that this plasmid codes for its own replication protein. Accordingly, electrophoretic analysis of radiolabelled plasmid-encoded proteins showed that a 36 kDa protein, which was essential for plasmid replication, was encoded within the pMJ101 replication region. The expression of this protein was correlated with the ability of different recombinant plasmids harbouring this pMJ101 DNA region to replicate in an *E. coli* PolA deficient strain. Interestingly, pMJ101 did not show detectable homology when tested with *rep* probes representing all known plasmid incompatibility groups. Furthermore, a recombinant derivative harbouring the pMJ101 replication region proved to be compatible with the *V. anguillarum* 775 pJM1 plasmid (Bidinost *et al.*, 1999).

A 1.56 kb fragment harbouring the pMJ101 replication region (Bidinost *et al.*, 1999) contains an AT-rich region, 11 *dam*-methylation sites, of which five are within the putative *ori* region, and five copies of the 9 bp consensus sequence for DnaA binding (Fig. 15.6). The latter replication element binds DnaA purified from *E. coli*. A potential open reading frame encoding a hydrophilic protein with a predicted pI of 10.3 and an M_r of 33,826 Da is adjacent to the *ori* region. Although these properties are typical of DNA-binding proteins, no significant homology has been found between this predicted protein, named RepM, and other previously characterized proteins. Reverse transcriptase polymerase chain reaction analysis of total RNA demonstrated the presence of *repM* mRNA in *V. ordalii*. The major initiation site of this mRNA was located 187 nucleotides upstream of the GTG initiation codon. This transcription initiation site is preceded by putative –10 and –35 promoter sequences that control the expression of the *repM* replication gene (Fig. 15.6). Southern blot analysis of denatured and non-denatured DNA did not show the presence of single-strand DNA, indicating that a theta replication mechanism was involved in the maintenance of this plasmid (Bidinost *et al.*, 1999). In spite of these results, the role of pMJ101 in the *V. ordalii* life cycle and/or its virulence properties

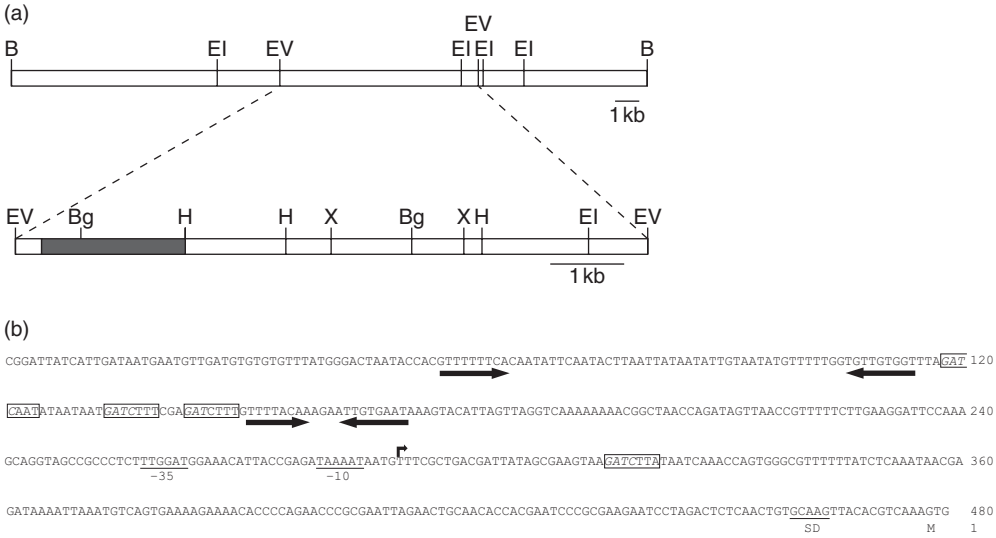


Fig. 15.6. (a) Physical and genetic map of pMJ101. Restriction endonuclease sites are shown for *Bam*HI (B), *Bgl*II (Bg), *Hin*dIII (H), *Eco*RI (EI), *Eco*RV (EV) and *Xba*I (X). The DNA fragment harbouring the minimal pMJ101 replication region is indicated by the shaded area. (b) Nucleotide sequence of the pMJ101 origin of replication. The location of the predicted Shine-Dalgarno (SD) and –10 and –35 promoter elements are indicated by underlined nucleotides. The main transcription initiation site is indicated by the bent arrow. Predicted GATC methylation sites are indicated in italics and the conserved 7 bp repeats containing them are shown within the open boxes. The horizontal arrows indicate the position and orientation of putative DnaA boxes. The first amino acid of RepM is shown below the last nucleotide triplet.

remains unknown. Therefore, the isolation of an isogenic cured derivative will be an invaluable tool to assess the function of this universal plasmid present in *V. ordalii* isolates. Alternatively, the biological role of pMJ101 could be predicted and elucidated once its complete nucleotide sequence is determined.

The disease

V. ordalii localizes more frequently in the muscle and skin, with bacterial colonies or aggregations that can replace large areas of host tissues, of chum salmon suffering naturally acquired vibriosis. This tissue tropism is different from that displayed by *V. anguillarum* (Schiewe, 1983; Ransom *et al*., 1984). *V. ordalii* also causes the necrosis and haemorrhaging of the surrounding tissues in some of the infected sites. All these findings indicate that this fish pathogen enters the host by invasion of the integument of the salmon host. Bacterial colonies are commonly found in loose connective tissues, the gills, throughout the digestive tract and in the pyloric caeca, an observation that suggests that the infection could also begin at these sites of the host. *V. ordalii* can be observed, although only occasionally, as microcolonies in spleen and liver, and low counts in blood may be detected during the initial stages of the infection. A similar pathology was observed during experimental waterborne infections of chum, coho or Chinook salmon exposed to a large number of bacterial cells. Juvenile salmon exposed to *V. ordalii* by parenteral challenge developed a systemic infection and the bacterium was recovered from blood, kidney, liver and spleen immediately after the infection (Schiewe, 1983). However, the number of bacteria in the liver declined after 1 h and then increased after 22 h post-infection. After 6 days of infection, bacterial numbers were high in all the organs, with 100%

mortality of infected salmon. These observations demonstrate the value and significance of artificial infection of juvenile salmon when used as an experimental model to study the bacterial mechanisms and virulence factors involved in the pathogenesis of the fish infections caused by *V. ordalii*.

Virulence factors

Overall there is a limited amount of information on the nature of the virulence factors involved in the pathogenesis of the infections this bacterium causes in salmon. There is an apparent correlation between the presence of *V. ordalii* in the host blood and an apparent decrease in white blood cell counts in moribund fish during the infection (Harbell *et al.*, 1979; Ransom *et al.*, 1984). This observation suggests the production of a leucocytolytic factor, which may play an important role in the pathogenesis of the vibriosis syndrome caused by this bacterium. Other factors can also play an important role, such as the ability of this pathogen to resist the bactericidal activity of normal non-immune rainbow trout serum, which has been correlated with the virulence of this bacterium (Trust *et al.*, 1981). *V. ordalii* strains also agglutinate trout and human erythrocytes, as well as yeast cells, although some discrepancies have been reported, due probably to technical artefacts (Trust *et al.*, 1981; Larsen and Mellegaard, 1984). The ability of *V. ordalii* to agglutinate different eukaryotic cell types suggests that it attaches to and interacts with the host cells. However, a correlation between the agglutination phenotype and the virulence properties could not be determined. Furthermore, the potential role of these agglutinins during the onset and progress of infections in salmonids remains to be tested.

Vibrio salmonicida

The bacterium

V. salmonicida, a psychrophilic bacterium that causes coldwater vibriosis in Atlantic salmon (*Salmo salar*), rainbow trout (*O. mykiss*) and cod (*Gadus morhua*) (Bruno *et al.*, 1986; Egidius *et al.*, 1986; Wiik *et al.*, 1989a; Toranzo *et al.*, 2005), is a facultative anaerobe motile rod. It possesses several polar flagella (Fig. 15.7) (Egidius *et al.*, 1986). It has been proposed recently that this bacterium be reclassified as *Aliivibrio salmonicida* (Urbanczyk *et al.*, 2007). When isolated from fish, this bacterium shows a high degree of pleomorphism. *V. salmonicida* is a halophilic bacterium that grows in the presence of 0.5–4% NaCl, with an optimum salt concentration of 1.5% (Egidius *et al.*, 1981). The bacterium is psychrophilic and can grow between 1 and 22°C, with an optimum temperature of 12–15°C. Colonies on nutrient agar supplemented with blood are small and greyish, showing no pigmentation (Egidius *et al.*, 1986). *V. salmonicida* is non-haemolytic when grown on solid media containing either human or sheep blood. It can be isolated from sediments obtained from farms in which fish have had *V. salmonicida* vibriosis, but have been disease free for up to 7 months prior to the study (Hoff, 1989), and is also isolated from marine sediments from disease-free farms. However, this bacterium has not been detected in sediments of regions not influenced by fish farming. Since *V. salmonicida* has been isolated from faeces of experimentally infected fish, these results suggest that *V. salmonicida* could reach the marine sediments through the fish faeces and remain there for long periods of time (Enger *et al.*, 1989). In addition, healthy fish can be carriers and they contribute to its spread in the marine environment.

V. salmonicida is serologically distinct from *V. anguillarum*. No immunological cross-reaction was detected with three strains of *V. anguillarum* using rabbit polyclonal antibodies raised against *V. salmonicida* (Egidius *et al.*, 1986). However, Enger *et al.* (1989) showed that polyclonal antibodies against *V. salmonicida* could cross-react with *V. anguillarum* 775 and *V. ordalii* PT1 strains isolated from Atlantic cod (*G. morhua*), as well as against uncharacterized strains isolated from marine

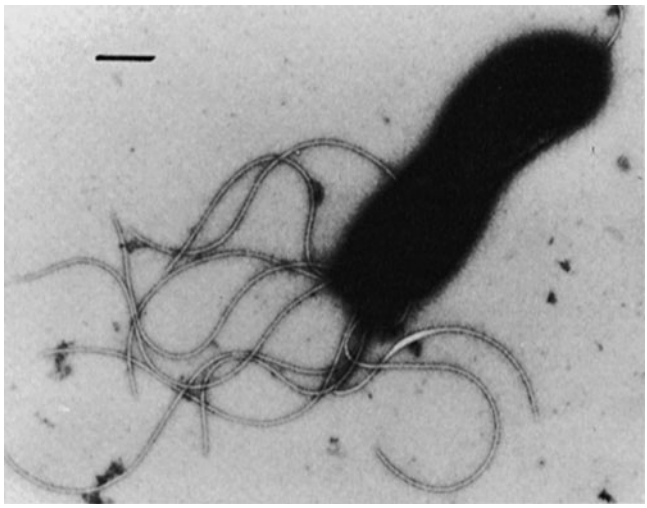


Fig. 15.7. Electron micrograph of *V. salmonicida* showing a polar tuft of sheathed flagella. × 7120. Bar, 1 μm. (From Egidius *et al.*, 1986, with permission from the authors and the American Society for Microbiology.)

sediments. Conversely, the utilization of monoclonal antibodies allowed the detection of *V. salmonicida* only (Enger *et al.*, 1989). The dominant antigen of *V. salmonicida* is a hydrophobic protein, known as VS-P1, located on the bacterial cell surface (Espelid *et al.*, 1987).

V. salmonicida strains are differentiated by their plasmid profiles (Sørum *et al.*, 1988, 1990; Wiik *et al.*, 1989a). Common plasmid-isolation techniques such as the alkaline extraction procedure described by Birnboim and Doly (1979) or that described by Kado and Liu (1981) gave poor results, however, when applied to *V. salmonicida* (Wiik *et al.*, 1989a). An alternative method that did not include the alkaline denaturation step proved to be adequate for plasmid isolation from this bacterium (Orberg and Sandine, 1984; Wiik *et al.*, 1989a). Plasmids of 170, 61, 24, 10, 3.4 and 2.8 MDa were detected in *V. salmonicida* isolated from diseased cod and Atlantic salmon (Sørum *et al.*, 1988; Wiik *et al.*, 1989b). Certain plasmid profiles were characteristic of strains isolated in different geographical regions on the Norwegian coast (Table 15.3). This table shows that the 61 MDa plasmid is present only in strains isolated from northern Norway, while the 21 MDa plasmid seems to be ubiquitous. The relationship among these plasmids was studied by DNA–DNA hybridization. The only homology found was between the 24 and the 2.8 MDa plasmids.

Epidemiological studies based on plasmid profiles suggest that *V. salmonicida* is transmitted from cod to Atlantic salmon and vice versa in fish farms in northern Norway (Sørum *et al.*, 1990). No correlation could be found between the plasmid profiles and the LD_{50} of the strains analysed. Valla *et al.* (1992) has analysed the 10 MDa plasmid pVS1 present in the strain TEO83.00. This study shows that this plasmid harbours repeated sequences, as well as sequences homologous to other plasmids present in other isolates of *V. salmonicida*. A curing method for pVS1 was developed based on plasmid incompatibility, since the utilization of chemical agents was ineffective. A recombinant derivative of the IncQ plasmid pPV14 harbouring pVS1 incompatibility determinants (pPV14.16) was introduced by conjugation into the *V. salmonicida* TEO83.001 strain. Selection after the conjugation for the pPV14.16 incoming plasmid resulted in the loss of

Table 15.3. Plasmid patterns of *V. salmonicida* strains isolated from different regions of the Norwegian coast (data taken from Sørum *et al.*, 1990).

Plasmid pattern	Geographical region of Norwegian coast[a]		
(MDa)	Northern	Central	Western
61, 21, 3.4, 2.8	14		
61, 21	1		
21, 3.4, 2.8	20	1	3
21, 3.4	13	38	24
21	3		6

[a]Number of *V. salmonicida* strains isolated along the Norwegian coast containing plasmids of the indicated sizes.

pVS1. Further growth of this exconjugant strain in the absence of selective pressure resulted in a plasmid-free derivative, due to the instability of pPV14.16 in *V. salmonicida* TEO83.001. Experimental infections and serotype analysis of the wild type and the cured derivative demonstrated that there was not a significant correlation between the plasmid content and the virulence of *V. salmonicida* TEO83.001. Furthermore, the curing of this plasmid did not affect the metabolic properties of this strain.

The largest plasmid, with a size of 170 MDa, has been characterized and found to carry a tetracycline resistance gene (Sørum *et al.*, 1992). In Norway, oxytetracycline is used for treatment against *V. salmonicida* and no tetracycline-resistant *V. salmonicida* have been detected for several years. However, several resistant strains have emerged (Sørum *et al.*, 1992). For one strain, the tetracycline-resistance gene was identified and cloned from the 170 MDa plasmid named pRSV1. By hybridization analysis, it was found that it has homology to the *tetA(E)* gene isolated from a human *E. coli* isolate and strains of *Aeromonas hydrophila* (Sørum *et al.*, 1992). The expression of this gene, encoding a 26.5 kDa protein, appears to be regulated by the concentration of tetracycline in the growth medium. Removal of a DNA fragment rendered expression of this gene constitutive, suggesting the presence of a regulatory system as has been described already for the *E. coli tetA(E)* gene (Tovar *et al.*, 1988). Later, Andersen and Sandaa (1994) showed, using DNA probes for the tetracycline-resistance determinants A–E, that E was the most dominating determinant detected in polluted and unpolluted marine sediments isolated from Norway and Denmark. These investigators suggested that the altered distribution of antibiotic resistance observed during this study might be the consequence of the increased consumption of tetracycline for human and veterinary purposes and the disposal of faecal-contaminated water in the marine environment, along with the appearance and spread of antibiotic-resistant microorganisms.

The *V. salmonicida fur* gene has been cloned and characterized. Hybridization experiments showed that it was conserved among *V. salmonicida* isolates and other Vibrios (Colquhoun and Sørum, 2002). *V. salmonicida* was shown to produce one or more siderophores. Interestingly, although in iron-limited media optimal growth temperature is 12°C, significant quantities of a hydroxamate siderophore, later identified as the cyclic dihydroxamate bisucaberin, were produced only at 10°C or below (Colquhoun and Sørum, 2001; Winkelmann *et al.*, 2002).

Although phenotypically distinct, comparative studies of the 16S rRNA gene sequences have revealed a close phylogenetic relationship between *V. salmonicida* and *V. fischeri* and *V. logei*, the luminous marine bacteria (Wiik *et al.*, 1995; Fidopiastis *et al.*, 1998, 1999). Further studies showed that *V. salmonicida* cultures produced detectable luminescence if they were supplemented with an aliphatic aldehyde (Fidopiastis *et al.*, 1999). Bacteria that carry the genes for luciferase but do not produce a detectable level of light in culture have been referred to as cryptically bioluminescent, and *V. salmonicida* is one of the best-studied cases (Fidopiastis *et al.*, 1999). Luminescence is also expressed when the cells are exposed to *N*-3-oxohexanoyl homoserine lactone, the signal molecule that is in part

responsible for quorum sensing and the induction of luminescence in *V. fischeri* (Miller and Bassler, 2001). However, *V. salmonicida* induces luciferase production at a much lower level as compared to *V. fischeri*. Genetic analysis showed that *V. salmonicida* had both a novel arrangement and a different number of homologues of the *luxR* and *luxI* quorum-sensing regulatory genes and the reduced levels of luminescence might be due to antisense transcripts (Nelson *et al.*, 2007). Nelson *et al.* (2007) found a link between luminescence and the virulence of *V. salmonicida*.

The *V. salmonicida* catalase has been the subject of detailed studies, culminating with its crystal structure resolved at 1.9 Å (Riise *et al.*, 2007). It was found that this enzyme had features that revealed adaptation to cold temperatures and it was two times more catalytically efficient than the catalase from the mesophilic human pathogen *Proteus mirabilis* (Lorentzen *et al.*, 2006). The *V. salmonicida* endonuclease I (EndA), a periplasmic or extracellular enzyme present in many different Proteobacteria, also showed typical cold-adapted features such as lower unfolding temperature, lower temperature optimum for activity and higher specific activity when compared with that of *V. cholerae* (Altermark *et al.*, 2007; Niiranen *et al.*, 2008). The chemical structure of the oligosaccharide part of *V. salmonicida* lipopolysaccharide serotype C2, isolated from Atlantic cod, has been determined recently by NMR spectroscopy and mass spectrometry (Bogwald and Hoffman, 2006).

The disease

Hitra disease appeared in 1977 and occurred for the first time on a large scale in 1979 in fish farms on the Norwegian island of Hitra. Since then it has devastated fish farms located along the western and northern Norwegian coast (Egidius *et al.*, 1981). However, single outbreaks have also been reported in Scotland (Bruno *et al.*, 1985), the Faroe Islands (Dalsgaard *et al.*, 1988) and in New Brunswick and Nova Scotia, Canada (referenced in Sørum *et al.*, 1992). This disease affects mainly fish farms with Atlantic salmon and occasionally with rainbow trout. *V. salmonicida* has also been described as the aetiological agent of cold-water vibriosis affecting farmed cod in Norway (Sørum *et al.*, 1990). Hitra disease occurs mainly in late autumn, winter or early spring (Egidius *et al.*, 1981). The characteristics of this disease, also known as haemorrhagic syndrome, are anaemia and haemorrhage with a generalized septicaemia, presenting large amounts of bacterial cells in blood of moribund or recently dead fish. Haemorrhage is found mainly in the integument surrounding the internal organs of the fish (Poppe *et al.*, 1985; Egidius *et al.*, 1986). However, in some cases of cod infections, some differences were found in the pathology. The infected cod fry show cataracts, cranial haemorrhage and splenomegaly, signs that are closer to those observed in cod when infected with *V. anguillarum* (referenced in Sørum *et al.*, 1990).

A recent study was conducted to investigate the effect of fish skin mucus on the global protein expression of *V. salmonicida* (Raeder *et al.*, 2007). Cells were cultured in synthetic media with and without the addition of fish skin mucus. Increased levels of proteins involved in motility (flagellar proteins FlaC, FlaD and FlaE), oxidative stress responses (peroxidases TPx, Grx and AhpC) and general stress responses (chaperons DnaK and GroEL) were found in cells growing in the presence of mucus (Raeder *et al.*, 2007). However, the addition of mucus to the culture medium did not alter the growth rate of *V. salmonicida* significantly. The changes in *V. salmonicida* protein expression may be due to the presence of a protein that potentially possesses catalytic activity against DNA and a protein with iron chelating activity in fish skin mucus (Raeder *et al.*, 2007).

Vibrio vulnificus

The bacterium

V. vulnificus, also known as *V. anguillicida*, is capable of causing disease in cultured eel

(*A. japonica*) (Nishibuchi *et al.*, 1979, 1980). It was isolated during outbreaks of vibriosis in eels in Japan (Muroga *et al.*, 1976) and the UK (Austin, 1987). Comparison among clinical, environmental and eel isolates of *V. vulnificus* showed that although highly related, the eel isolates had distinct phenotypic, cultural and serological properties. Furthermore, only the eel isolates were pathogenic to eels as well as to mice, while typical *V. vulnificus* isolates were pathogenic to mice only (Tison *et al.*, 1982). These differences indicated that the eel isolates belonged to a different biogroup, and it was thus proposed that the *V. vulnificus* strains similar to those isolated from diseased eels should be classified as *V. vulnificus* biogroup 2, keeping the designation of *V. vulnificus* biogroup 1 for clinical and environmental strains (Tison *et al.*, 1982). Because of these differences and its O serogroup homogeneity independent of their geographical origin, this is now considered a new biotype of *V. vulnificus*, which is well adapted to eels, and therefore classified as serotype E (Biosca *et al.*, 1997; Toranzo *et al.*, 2005). Therefore, the confirmation of this bacterium as the cause of vibriosis must include its identification using an agglutination test with specific antibodies, together with the use of selective media as proposed before (Marco-Noales *et al.*, 2001). This fish pathogen can also be identified using an ELISA for the haemolysin, avoiding the lengthy and labour-intensive biochemical assays used for its identification (Parker and Lewis, 1995). Molecular-based methods also have been developed, including a nested polymerase chain reaction (PCR) method for rapid and sensitive detection of *V. vulnificus* in fish and environmental specimens, using rRNA-targeted oligonucleotide probes specific for *V. vulnificus* or primers specific for the *V. vulnificus* cytolysin gene (Aznar *et al.*, 1994; Arias *et al.*, 1995; Coleman *et al.*, 1996; Toranzo *et al.*, 2005).

The disease

V. vulnificus biogroup 2 survives in brackish water and attaches to eel skin, suggesting that water and infected fish act as reservoirs (Amaro *et al.*, 1995). In addition, the survival of this bacterium in the environment and the spread of the disease depend on the temperature and salinity of the water (Kaspar and Tamplin, 1993; Amaro *et al.*, 1995).

The ability of this salt-requiring bacterium to cause disease is associated with phenotypic properties such as colony morphology and the presence of a capsule (Yoshida *et al.*, 1985; Simpson *et al.*, 1987; Wright *et al.*, 1990; Amaro *et al.*, 1994, 1995). It has been suggested that the production of a capsule by *V. vulnificus* biotype 2 is essential for the survival of the pathogen in the marine environment and the colonization and invasion of the host. This conclusion is based on the fact that non-encapsulated cells can cause vibriosis in eels when injected intraperitoneally, although pure cultures of encapsulated cells have always been recovered from diseased fish (Biosca *et al.*, 1993). Other potential virulence factors include the secretion of extracellular cytotoxic and cytolytic factors (Kreger and Lockwood, 1981; Gray and Kreger, 1985; Amaro *et al.*, 1994), an elastolytic protease (Kothary and Kreger, 1987), an extracellular phospholipase (Testa *et al.*, 1984), resistance to phagocytosis and the bactericidal effects of sera (Johnson *et al.*, 1984; Yoshida *et al.*, 1985). The availability of iron was also correlated with the virulence of *V. vulnificus* (Wright *et al.*, 1981; Jayalakshmi and Venugopalan, 1992; Amaro *et al.*, 1994). Clinical and environmental isolates of *V. vulnificus* can use haemin-containing compounds such as haemoglobin, haemoglobin-haptoglobin, haemin and haemin-albumin to reverse iron limitation (Zakaria-Meehan *et al.*, 1988; Nishina *et al.*, 1992; Simpson and Oliver, 1993). However, the utilization of haemoglobin-haptoglobin and haemin-albumin is correlated with the expression of a proteolytic activity that is regulated positively by the presence of either haemin or haemoglobin in the medium (Simpson and Oliver, 1993). Simpson and Oliver (1983) showed that this bacterium secreted iron-chelating compounds when cultured under iron deficiency. The siderophore

vulnibactin was purified and characterized as a molecule containing 2,3-dihydroxybenzoic acid, salicylic acid, L-threonine and norspermidine (Okujo *et al.*, 1994), a pathway that seemed to involve the expression of at least two iron- and Fur-regulated, non-ribosomal peptide synthetases (Kim *et al.*, 2008). This iron chelator is recognized and internalized by VuuA, the outer membrane receptor whose production is regulated by iron as well as the CRP protein complex (Webster and Litwin, 2000; Choi *et al.*, 2006). In addition to expressing the vulnibactin-mediated system, *V. vulnificus* is capable of using siderophores produced by other microorgansims such as aerobactin and deferoxamine (Tanabe *et al.*, 2005; Kim *et al.*, 2007), a property that may enhance the persistence of this fish pathogen in different iron-restricted ecological niches. The importance of iron in the pathobiology of *V. vulnificus* was evidenced by a recent global gene expression analysis, which revealed the differential expression under iron-rich and iron-limiting conditions of genes such as those coding for iron acquisition, porins and amino acid biosynthesis (Alice *et al.*, 2008). It is apparent that some of these differentially expressed genes potentially are involved in the pathogenesis of the infections this bacterium causes in vertebrates.

Severe human infections caused by *V. vulnificus* biogroup 1 have been reported (Blake *et al.*, 1980), and environmental studies have demonstrated that this bacterium is widespread along coasts (Oliver *et al.*, 1983). A case of human infection caused by a *V. vulnificus* strain very similar to the eel isolates has been described (Veenstra *et al.*, 1992). Although this human isolate was indole-negative, the main characteristic of biogroup 2, other reactions such as the ornithine decarboxylase test and growth at 42°C were different from those described by Tison *et al.* (1982). The infection seemed to be caused from contact with an infected eel through an open wound (Veenstra *et al.*, 1992). It has been reported that *V. vulnificus* fatal infections are also associated with eating contaminated raw or undercooked seafood, particularly raw oysters (Mascola *et al.*, 1996). This study demonstrated that immunocompromised patients and persons with chronic liver disease were at increased risk. A third biotype, which could be differentiated from biotypes 1 and 2, was isolated from human infected wounds after contact with *Tilapia* (Bisharat *et al.*, 1999). A more detailed description of the bacterial and host factors involved in the pathogenesis of human infections caused by *V. vulnificus* can be found in recent reviews (Borenstein and Kerdel, 2003; Oliver, 2005, 2006; Bross *et al.*, 2007).

Treatment and Prophylaxis

It is apparent that the need for protein from marine resources will increase in the future, with aquaculture playing a central role in this process. However, a more intense use of fish farming will be associated with significant challenges, such as the prevention and treatment of marine infections that have a profound impact in the food industry.

One alternative to address the aforementioned issues is the utilization of antimicrobial compounds to control bacterial infections, such as vibriosis, in farmed fish. Antibiotics and other chemotherapeutic agents have been used as feed additives or added directly to the water to prevent and treat vibriosis. In Japan, ampicillin, chloramphenicol, nalidixic acid derivatives, nitrofuran derivatives, sulfonamides and trimethoprim have been routinely used to treat vibriosis (Aoki *et al.*, 1984). However, the use of these compounds has resulted in drug-resistant strains (Watanabe *et al.*, 1971; Shotts *et al.*, 1976; Aoki *et al.*, 1977, 1979, 1980; Hayashi *et al.*, 1982; Toranzo *et al.*, 1984). Up until 1977, tetracycline-resistant isolates were recovered from cultured ayu (*P. altivelis*), when the use of this antibiotic was discontinued. Analysis of the resistance profile of *V. anguillarum* recovered since 1978 has shown that only one isolate is resistant to tetracycline (Aoki *et al.*, 1984), demonstrating a correlation between the use of tetracycline and appearance of

resistance. Molecular and genetic analyses show that the genes encoding antibiotic resistance are often found in plasmids. Furthermore, in some cases, these plasmids were shown to be conjugative (Aoki *et al.*, 1984; Toranzo *et al.*, 1984). Further studies are needed to demonstrate whether these genes are also present in transposable elements or integrons (Craig *et al.*, 2002).

It is apparent from all these studies that the main drawback of this approach to control and prevent vibriosis is the selection for resistant strains and the spreading of antibiotic resistance determinants among fish pathogens, as well as pathogens that have the potential to affect other important human food sources. All these negatives effects do not take into account the negative impact these antimicrobial compounds have on the natural environment and a wide range of biological systems.

To avoid some of the aforementioned problems while maintaining a dependable food source, the modern aquaculture industry has adopted a wide range of control measures, farming practices and science-based approaches that have resulted in more healthy and reliable fish products (Chinabut and Puttinaowarat, 2005; Corsin *et al.*, 2007). These practices include alternatives to the use of antibiotics to control infectious fish diseases, such as the development and utilization of vaccines and probiotics that prevent the outbreaks of bacterial infections in cultured fish.

Vaccination has been one of the most effective tools in controlling diseases in the fish industry, particularly that related to salmon farming (Gudding *et al.*, 1999). The overall goal of this approach is the immunoprophylaxis of fish with bacterial antigens administered by injection, immersion or oral administration, with each of these methods having their advantages and disadvantages (Gudding *et al.*, 1999). Most of the vaccination methods are based on the administration of aqueous bacterial products produced in the laboratory and inactivated before administration, known as bacterins, while some other alternatives include live and attenuated vaccines or vaccines based on DNA technology. Traditional vaccines consist of formalin-killed *V. anguillarum* or bacterial membrane components (Agius *et al.*, 1983) and give the best protection when administered by intraperitoneal injection rather than by immersion or oral administration (Kawano *et al.*, 1984; Ward *et al.*, 1985), but the former method has limitations and for practical reasons the other approaches are currently used. Attempts to develop a live vaccine using avirulent mutants have used transposition mutagenesis. Norqvist *et al.* (1989) constructed avirulent mutants capable of inducing protective immunity after bath vaccination. Avirulent strains were constructed by curing of the virulence plasmid pJM1 (Crosa, 1980), by performing other genetic manipulations of this plasmid such as deletion of the iron-uptake region (Walter *et al.*, 1983) and mutagenesis by either transposition (Tolmasky *et al.*, 1988a) or *in vitro* insertion (Singer *et al.*, 1991). In this latter case, the avirulent derivative persisted in inoculated fish. The mutants could be isolated 9 days post-inoculation, suggesting that these avirulent *V. anguillarum* derivatives could be good candidates for a live attenuated vaccine. Bacterin preparations including serotype O1, a mixture of serotypes O1 and O2a or the GAVA-3 bacterin, which covers serotypes O1, O2a and O2b, have been used successfully to prevent vibriosis caused by *V. anguillarum* (Newman, 1993; Toranzo *et al.*, 2005). Also available from commercial sources are polyvalent oil-based vaccines that protect Nordic cultured salmonids from infections caused by several microbial pathogens, including all three vibrios described above (Toranzo *et al.*, 1997). More recently, Spanish investigators have developed an efficient bacterin known as Vulnivaccine for the treatment of fish infections caused by *V. vulnificus* (Fouz *et al.*, 2001). However, this vaccine has proved to be efficient only in infections caused by serotype E strains of this fish pathogen (Fouz and Amaro, 2003).

Although significant progress has been made in the past few years, the successful application of vaccines to prevent and control fish infections depends on a better

understanding of the fish immune system and its interaction with bacterial pathogens and their virulence determinants. In addition, the potential value of alternatives such as the utilization of immunostimulants and the application of probiotics to control fish infections are worth exploring further. This type of strategy to combat vibriosis has been proposed based on observations that bacteria of the normal gut flora produce inhibitory substances (Lemos *et al.*, 1985; Onarheim and Raa, 1990; Westerdahl *et al.*, 1991). After studying more than 400 intestinal isolates from turbot (*Scophthalmus maximus*, L.), Westerdahl *et al.* (1991) found that 28% of those isolates exhibited inhibitory effects against *V. anguillarum*. More recently, it has been shown that bacteria recovered from the digestive microbial flora of rainbow trout and carp are effective probiotic tools to control infections caused by several fish pathogens, including *V. anguillarum* and *V. ordalii*, by positively affecting the nutrition, production of bacterial inhibitory compounds and stimulation of the immune system of the fish host (Brunt *et al.*, 2007). These results demonstrate the value of using live and bacterial cells and their products to stimulate fish innate defences to prevent infections that have severe effects on the fish industry. Equally relevant is the better understanding of the virulence repertoire of fish bacterial pathogens, which could be attained with classical as well as more modern approaches such as bacterial genomics. Crosa *et al.* are developing such an approach (work in progress), with the ultimate goal of gaining a more comprehensive understanding of the molecular and genetic host–pathogen interactions that result in vibriosis caused by *V. anguillarum* and *V. ordalii*.

Conclusions and Recommendations for Future Studies

Although significant advances in basic knowledge and development of therapeutic tools have been made in the past few years, it is apparent that vibriosis still remains an important cause of fish infectious diseases. It is also evident that the combination of classical tools, such as the development of vaccines using killed bacterial pathogens, with more modern approaches of detection, such as multiplex PCR, has made important strides in the fish industry as well as in basic science related to the pathogenesis of fish diseases. These encouraging outcomes reinforce the concept that modern approaches, such as genomics, global gene analysis and proteomics, have a great potential for providing a better understanding of the pathobiology of the bacterial agents responsible for vibriosis. Such knowledge should facilitate the development of more convenient diagnostic tools, as well as preventing and/or treating vibriosis in a more rational manner. Furthermore, the understanding of the host response to the infections caused by the bacterial agents described in this chapter could help in the development of better and more convenient approaches in aquaculture and perhaps the engineering of fish that are more resistant to bacterial infections. All these approaches should bring the relevance of bacterial fish diseases closer to that of human infections, not only because of the potential relationships in the response to bacterial infections of these two types of vertebrate hosts but also because of the relevance of fish in the environment as well as being an invaluable human food resource.

Acknowledgements

The experiments from Dr Crosa's laboratory reported here were supported by Public Health Grant AI19018. Public Health AI070174 and DE13657 grants, NSF 0420479 grant and Miami University research funds supported the research conducted in Dr Actis's laboratory. California State University Fullerton intramural grants and Public Health Grant No. AI047115 supported the research conducted in Dr Tolmasky's laboratory.

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16 Flavobacterial Diseases: Columnaris Disease, Coldwater Disease and Bacterial Gill Disease

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Introduction

There are three primary bacterial species within the genus *Flavobacterium* that cause significant diseases to hatchery-reared and wild populations of freshwater fish. *F. psychrophilum*, which causes bacterial coldwater disease, is particularly problematic to trout and salmon species. The bacterium was described initially and recovered in 1948 from a die-off in coho salmon (*Oncorhynchus kisutch*; Borg, 1960). The second bacterium is *F. columnare*. It is the disease agent of columnaris disease, which affects many coolwater and warmwater fish species. Columnaris disease is a significant problem worldwide and is particularly problematic for important aquaculture species, a variety of aquarium species and free-ranging fish where outbreaks are common. Davis (1922) first described columnaris disease that was causing outbreaks of mortality to a variety of warmwater fish in the Mississippi River Valley (USA). Although a causative agent for the disease was not isolated at that time, the disease was reported based on observations of wet mount preparations from skin scrapings that showed numerous slender bacterial rods that clumped together to form columnar-shaped cellular masses; hence the name, columnaris disease. Isolation of the causative bacterium was by Ordal and Rucker (1944) from sockeye salmon (*O. nerka*). Co-infections of *F. psychrophilum* or *F. columnare* (Hawke and Thune, 1992) with other fish pathogens are not uncommon. Bacterial gill disease (BGD) occurs in cool- and coldwater fish and is predominantly a problem among hatchery-reared juvenile salmonid species (Bullock, 1972, 1990; Daoust and Ferguson, 1983; Schachte, 1983; Farkas, 1985). This disease is considered to be almost exclusively of cultured fish and is not recognized as a significant problem in wild fish. BGD was first reported by Davis (1926); however, it was not until 1978 that the causative agent of disease was isolated (Kimura *et al.*, 1978). Wakabayashi *et al.* (1989) conducted studies on BGD isolates from various origins and characterized the disease-causing bacterium, naming it *F. branchiophila*; however, the name was changed to *F. branchiophilum* to recognize proper nomenclature (von Graevenitz, 1990).

Combined, mortality caused by these three major pathogens arguably affects the broadest host range and encompasses the largest geographic area of any bacterial fish pathogen. This is because of their presumed ubiquitous nature and the broad range of water temperatures in which they persist.

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Most, if not all, cultured freshwater fish species are affected by at least one of these pathogens.

The Disease Agents

Flavobacterium psychrophilum

Bacterial coldwater disease is caused by the Gram-negative bacterium *F. psychrophilum* (formerly *Cytophaga psychrophila*) and *Flexibacter psychrophilus* (Bernardet and Grimont, 1989; Bernardet *et al.*, 1996). Microscopic examination of coldwater disease infected tissue material reveals long, thin, rod-shaped cells typically in a size range of 0.75–1.0 µm wide by 3–5 µm long (Fig. 16.1). Some cells may be attached end-to-end and consequently will appear to be longer. Several disease forms and clinical disease expressions occur with bacterial coldwater disease, all of which are attributed to *F. psychrophilum*. In addition to the common form of coldwater disease, low-temperature disease, fry mortality syndrome (not to be confused with thiamine deficiency induced mortality syndrome in salmon), tail rot, peduncle disease and 'atypical' coldwater disease are other names for the disease.

Rainbow trout fry syndrome is a serious disease to young fish and may result in a high percentage of deaths in fish populations (Lorenzen *et al.*, 1991; Bruno, 1992; Santos *et al.*, 1992; Toranzo and Barja, 1993). Daskalov *et al.* (2000) noted the similarities in pathologies of rainbow trout fry syndrome with those caused by feeding poor conditioned diets. They demonstrated a relationship in dystrophic changes to rainbow trout caused by feeding a diet that contained elevated oxidized lipids with the colonization of *F. psychrophilum* and the development of coldwater disease. Tail rot

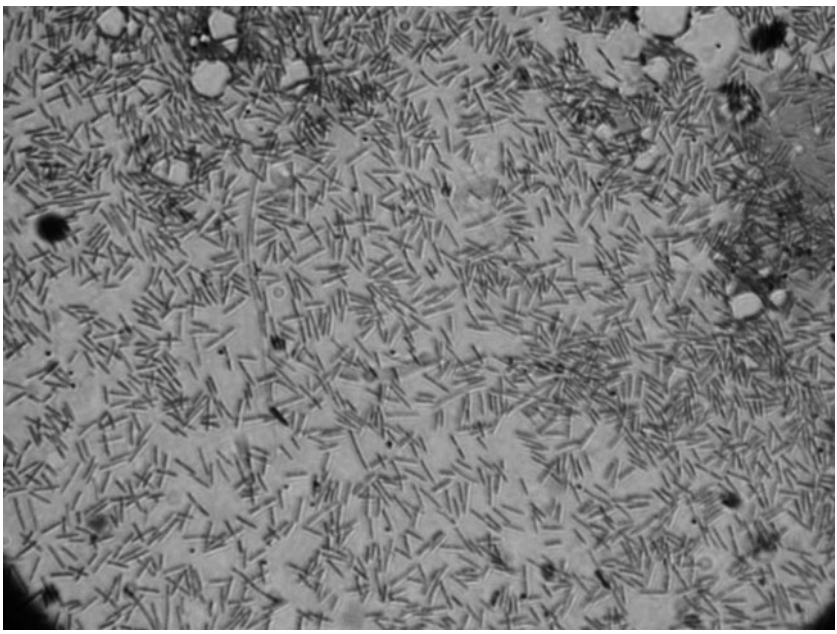


Fig. 16.1. Simple stain (crystal violet; 1000×) of *Flavobacterium psychrophilum* cells. Lesion material smear from an open lesion from rainbow trout (*Oncorhynchus mykiss*) affected with coldwater. Photomicrograph courtesy of Vermont Fish and Wildlife Department, Waterbury, Vermont (USA).

and peduncle disease are general lesions in (chronic) coldwater disease. Although various sizes of fish are affected, young fish are particularly susceptible (Holt, 1987; Toranzo and Barja, 1993; Brown *et al.*, 1997). Disease typically occurs at water temperatures of 12–16°C and is most prevalent and serious at about 10°C and below (Holt, 1987). These preferred water temperatures generally confine the disease to colder or northern climate locales.

Flavobacterium columnare

The causative bacterium of columnaris disease is *F. columnare* (Bernardet *et al.*, 1996), formerly *Chondrococcus columnaris*, *C. columnaris* and *F. columnaris*. Gram-stained preparations of cultures or infected tissues reveal long and thin Gram-negative rods (0.5–0.7 μm wide×3–5 μm long); some cells appear longer and may be attached end-to-end. Low magnification microscopy (e.g. 40×) of wet mount preparations from infected tissues such as gills or skin scrapings show bacterial cells that uniquely arrange into haystacks or columns (Fig. 16.2). Columnaris disease may cause both external pathology and systemic infections. *F. columnare* can be isolated readily from gills, body surface lesions and from internal organs (e.g. kidney, spleen and liver) of many of the fish. Although in the majority of clinical cases *F. columnare* can be isolated from both external and internal sites, Hawke and Thune (1992) have reported that in channel catfish (*Ictalurus punctatus*) the pathogen can be isolated as either an external or an internal infection from individual fish. Columnaris disease may be facilitated by physical injuries or abrasions to the skin/mucus that offer a good opportunity for *F. columnare* colonization (Vogel, 1958). Infections with *F. columnare* are often associated with external abrasions, such as to the mouth, fins and gills. Bacterial colonization of these infected sites also increases the

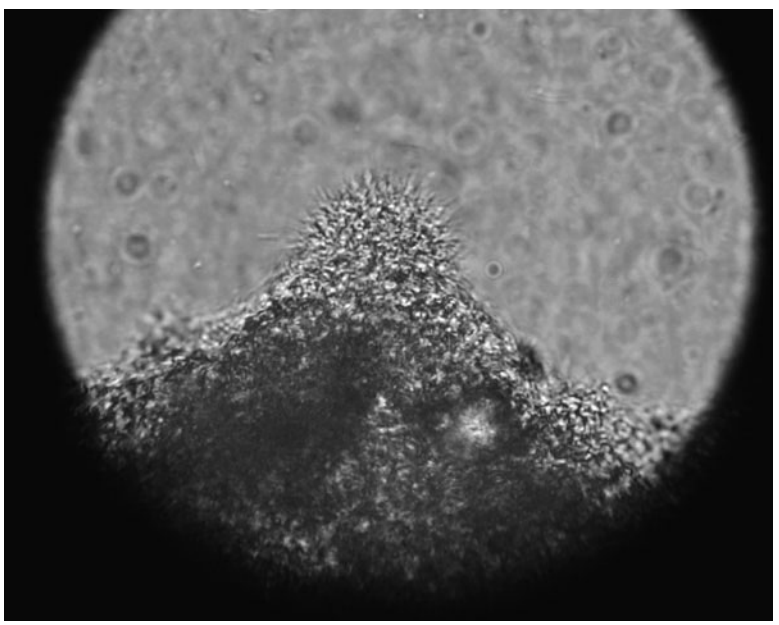


Fig. 16.2. Characteristic 'column' of *Flavobacterium columnare* cells. Wet mount observation (400×) of white crappie (*Pomoxis annularis*) infected gill tissue. Photomicrograph courtesy of Vermont Fish and Wildlife Department, Waterbury, Vermont.

number of cells in the water column which contribute to further disease spread. Until a successful treatment is administered, the morbidity and mortality in affected fish can be high.

Transmission is apparently horizontal through contact with infected fish or through exposure to bacteria in a water supply. As water temperatures increase, columnaris disease may develop in cultured fish that are reared in water from an open source, such as river water where *F. columnare* is present. This is especially true if the fish are exposed to stressors from crowding, reduced dissolved oxygen, handling or other factors, (Wakabayashi, 1991).

F. columnare has surface components that are important to establishing infections on external surfaces through attachment (Johnson and Chilton, 1966; Thune *et al.*, 1993). Extracellular proteases produced by *F. columnare* are important virulence factors involved in necrotic tissue pathology (Griffin, 1991; Bertolini and Rohovec, 1992). Various *F. columnare* isolates are uniform in their production of proteases that degrade gelatin, casein, haemoglobin, fibrinogen and elastin (Bertolini and Rohovec, 1992). Furthermore, increased iron (Fe^{2+}) enhances the virulence of *F. columnare* (Kou *et al.*, 1981). The pathogen is resistant to the bactericidal activity in normal catfish sera, although the classic antibody–complement cascade is also effective (Ourth and Bachinski, 1987). Additionally, *F. columnare* can lyse a number of competitive Gram-negative bacteria on fish surfaces (Pacha and Porter, 1968).

Flavobacterium branchiophilum

The causative bacterium of BGD was described by Wakabayashi *et al.* (1989), with isolates from the USA, Japan and Hungary (Kimura *et al.*, 1978; Wakabayashi *et al.*, 1980, 1989; Farkas, 1985). *F. branchiophilum* cells are Gram-negative, non-motile rods (0.5 μm wide × 5–8 μm long), which grow aerobically at 10–25°C. After 5 days of incubation at 18°C on cytophaga agar (Anacker and Ordal, 1959), colonies are 1.0–2.0 mm in diameter, pale yellow, smooth, with a slightly raised centre and non-spreading, entire border (Wakabayashi *et al.*, 1989). Catalase and cytochrome oxidase are produced, gelatin, casein and starch are utilized and acid is produced from glucose, fructose, sucrose, maltose, trehalose, cellobiose, melibiose, raffinose and inulin. The DNA G+C content ranges from 29 to 31 mol %. Isolates from Ontario, Canada, and Korea had the characteristic biochemical and physiological profiles; however, cell lengths of up to 15 μm were noted (Ostland *et al.*, 1994; Ko and Heo, 1997) and colony diameters were also greater (3–5 mm) after 6–7 days of incubation at 18°C (Ostland *et al.*, 1994). For many practitioners, routine primary isolation of *F. branchiophilum* on bacteriological media has proven to be extremely challenging, if not impossible. Because of this, and the relative ease of disease diagnosis using other techniques, culture of *F. branchiophilum* primarily is usually not attempted. Also, the bacterium is relatively slow growing and may be overgrown by other environmental bacteria that may colonize the gills.

F. branchiophilum is apparently ubiquitous in water supplies to many facilities. Resident fish at a hatchery and indigenous fish in water supplies also are probable reservoirs of infection. This was corroborated in a survey of North American hatcheries that rear rainbow trout. A significant association of mortality from BGD was found at hatcheries that had previous BGD outbreaks, and at facilities with indigenous fish in hatch house water supplies (Bebak *et al.*, 1997).

BGD only affects gills with no internal organ pathology. Fish suffer from impedance of respiration by large numbers of bacteria on the filaments and secondary lamellae, thus reducing gas exchange, and the resulting pathology. Transmission of *F. branchiophilum* is horizontal and the bacterium is highly contagious to fish. Attachment and colonization of trout gill tissues occurs in less than 1 h after experimental bath challenge to viable *F. branchiophilum*, and within a matter of hours after cohabitation with infected fish (Ferguson *et al.*, 1991; Ostland *et al.*, 1995). When rainbow

trout are exposed to *F. branchiophilum* by immersion, the extent of the resulting mortality is dependent on the number of viable cells in the water (Ostland *et al.*, 1995). Virulent and avirulent *F. branchiophilum* isolates adhere readily to gill tissues; however, only the virulent isolates are capable of colonization (Ostland *et al.*, 1995). Bullock *et al.* (1996) treated the water with ozone in a fluidized sand recirculation (closed) system to reduce heterotrophic bacteria significantly, and BGD epizootics were prevented. They (Bullock *et al.*, 1996) concluded that disease prevention was due, in large part, to the improved water quality from the ozone treatment process, which lowered the nutrient profile in the water and impeded growth of *F. branchiophilum*. Eliminating the numbers of *F. branchiophilum* cells from the water column removed the constant source for re-infection.

Geographical Distribution and Host Range

Diseases to fish caused by the *Flavobacterium* spp. are common and encompass a wide host range and geographic distribution. Many species worldwide are known to be susceptible to at least one of these pathogens. The geographic ranges of each of the *Flavobacterium* spp. generally are defined by water temperatures conducive to optimal bacterial growth. These bacteria usually are considered ubiquitous in freshwater aquatic environments and, to varying degrees, occur in conjunction with various host predisposing factors. The role of host predisposing factors is particularly relevant for BGD, which is recognized primarily as a problem of intensively cultured fish. Co-infections of *F. psychrophilum* and *F. columnare* with other pathogens or opportunistic bacteria, such as motile *Aeromonas* spp., and Oomycete infections are common.

Coldwater disease frequently occurs in wild and cultured salmonids, including Atlantic (*Salmo salar*), coho (*O. kisutch*) and Chinook salmon (*O. tshawytscha*), and rainbow (*O. mykiss*), brook (*Salvelinus fontinalis*), brown (*S. trutta*) and lake trout (*S. namaycush*) (Bullock, 1972; Bernardet and Kerouault, 1989; Santos *et al.*, 1992; Wiklund *et al.*, 1994; Bustos *et al.*, 1995; Schmidtke and Carson, 1995; Bernardet *et al.*, 1996; Cipriano *et al.*, 1996; Lorenzen and Olesen, 1997). The disease has also been reported from a variety of non-salmonid hosts, such as ayu (*Plecoglossus altivelis*), eel (*Anguilla anguilla*), carp (*Cyprinus carpio*), tench (*Tinca tinca*) and crucian carp (*Carassius carassius*) (Lehmann *et al.*, 1991; Wakabayashi *et al.*, 1994).

Columnaris disease affects coolwater and warmwater fish, typically in warm waters at 20–25°C and above; however, it is not too unusual to diagnose this disease in fish, including trout species, in water as cool as 12–14°C. Many cultured and free-ranging fish species residing in warmer temperatures are considered at risk of infection and possibly disease. Because of this wide host range and geographic distribution of disease, *F. columnare* is thought generally to be ubiquitous in temperate freshwater aquatic environments.

BGD affects cool- and coldwater fish and is particularly problematic for hatchery-reared juvenile salmonid fish (Bullock, 1972, 1990; Daoust and Ferguson, 1983; Schachte, 1983; Farkas, 1985). Brook trout and rainbow trout are particularly vulnerable. Mortality in diseased populations can rise quickly and the cumulative total percentage can be very high if the conditions are not improved and a treatment is not administered promptly. The disease generally is considered to be a problem to cultured fish and is not recognized as a problem to wild fish populations. It likely occurs worldwide wherever young salmonid fish are reared. In endemic areas, *F. branchiophilum* is considered to be a ubiquitous organism in freshwater aquatic environments, with BGD outbreaks often occurring with increased host stressors.

Economic Importance of the Disease

Diseases caused by *Flavobacterium* spp. result in significant losses of wild and

hatchery-reared populations of fish. An estimate of monetary losses by coldwater disease and columnaris disease to wild populations is difficult because of inherent problems with observing die-offs and recording accurate mortality data. Each of the three Flavobacterial diseases affects all age groups. Depending on production cycles in fish hatcheries and the time of the year of outbreaks, the total per cent mortality of small fish may be much greater than that for larger fish, simply due to the greater numbers of small fish after spawning and prior to stocking. When considering monetary losses to hatcheries, the cumulative losses are considerably higher for stocking-size and larger fish as a result of the long-term expenses of rearing these fish. Columnaris disease and enteric septicaemia of catfish (caused by *Edwardsiella ictaluri*) are responsible for the greatest losses to the catfish industry due to bacterial diseases. Undoubtedly, total economic losses to columnaris disease sustained by the aquaculture and pet fish industries are very significant – in numbers of fish lost to mortality, lesions that render fish non-marketable, in reduced growth rate and costs for treatments. In general, there are minimal social impacts associated with *Flavobacterium* spp. diseases in fish. None of the flavobacterial diseases of fish are known to produce diseases in other animals or humans. Losses to these diseases at fish hatcheries may reduce numbers of catchable-size fish available for stocking for sport fisheries purposes. Similarly, disease in wild populations may impact certain (USA) federally listed fish species. However, *Flavobacterium* spp. pathogenic to fish pose no obvious direct threat to the environment.

Diagnostic Methods

Common early disease signs in fish generally begin with lethargy and loss of appetite, and mortality that increases steadily in succeeding days. Diagnosis of coldwater disease can be based on case and facility histories, the water temperature, host(s) involved and clinical disease signs, and confirmation of *F. psychrophilum* as the causative agent from moribund or freshly dead fish. A chronic form of coldwater disease has been reported in salmonid fish, with fish displaying spiral or erratic swimming behaviour, blackened caudal (tail) area and spinal column deformities (Kent *et al.*, 1989; Blazer *et al.*, 1996). On initial observations, the signs and behaviour associated most often with this chronic form may be similar to those of whirling disease caused by the myxosporean parasite, *Myxobolus cerebralis*. However, bacterial coldwater disease may be diagnosed readily through bacterial culture and characterizations of *F. psychrophilum* from affected tissues including brain, kidney, liver and spleen.

Viable primary cultures of *F. psychrophilum* can be recovered from lesions and from the kidney, spleen, liver, brain and ascites. It is important to note that not all apparently affected fish will have a sufficient number of viable cells in internal tissues for culture. Recovery of the bacterium from lesions may be challenging due to the growth on primary isolation bacteriological media by other environmental bacteria and Oomycetes. Taking cultures from a greater number of fish will enhance the chance to recover the bacterium. It is possible to observe the bacterium on histologic slides and be unsuccessful in culturing the pathogen from that same tissue, or vice versa, particularly from asymptomatic fish with reduced infection levels. The pathology to fish caused by *F. psychrophilum* can be extensive, for example, focal necrosis in various organs, periostitis, osteitis, meningitis, ganglioneuritis and pyknotic nuclei (Kent *et al.*, 1989; Bruno, 1992). With chronic coldwater disease, masses of *F. psychrophilum* may be seen in the cranial area and anterior vertebra. Also, inflammation and cartilage necrosis along the vertebral column may be noted. Kent *et al.* (1989) correlated *F. psychrophilum* infections in the cranium and vertebrae of coho salmon with ataxia and abnormal swimming behaviour.

Primary culture plates can be inoculated using one of several techniques, such

as streak-plating or preparing a series of dilutions and drop-inoculating the medium surface to provide viable cell numbers (cfu/g). Homogenization of tissues prior to inoculations on to media may enhance recovery, especially from fish with low-level infections. Several bacteriological media may be used for primary culture of *F. psychrophilum*. Cytophaga medium (Anacker and Ordal, 1959) is probably the medium employed most frequently in the USA. It is not available as a prepared medium; it consists of 0.05% tryptone, 0.05% yeast extract, 0.02% sodium acetate and 0.02% beef extract. Other reduced nutrient concentration media have also been used (Anderson and Conroy, 1969; Holt, 1987; Bernardet and Kerouault, 1989; Cipriano *et al.*, 1996; Rangdale *et al.*, 1997a). Some authors report improved growth of *F. psychrophilum* after supplementing the medium with serum, a component typically used for slow-growing or fastidious bacteria that will grow on rich nutrient media. Lorenzen (1993) and Brown *et al.* (1997) incorporated 5.0% and 0.5%, respectively, of newborn calf serum. Obach and Baudin Laurencin (1991) supplemented cytophaga medium with 10% fetal calf serum for recovery of *F. psychrophilum* from rainbow trout. Daskalov *et al.* (1999) utilized cytophaga medium as a basal medium to which they added galactose, glucose, rhamnose and skimmed milk. Rangdale *et al.* (1997a) modified cytophaga medium by increasing the tryptone concentration tenfold (to 0.5%) and the beef extract to 0.05%. Various researchers have since used increased tryptone (to 0.5%) in cytophaga medium and excellent growth of laboratory cultures has been reported. Lorenzen (1993) underscored the importance of the brand of beef extract to culture *F. psychrophilum*, with optimal results using the semi-solid form. Kumagai *et al.* (2004) suggested the incorporation of 5μg/ml tobramycin to primary isolation plates to retard growth by contaminants.

The optimum incubation temperature for primary isolation and culture growth of *F. psychrophilum* is 15–16°C. Colonies on cytophaga agar have pale-yellow pigment and are 3–4 mm in diameter after 2–3 days of incubation. Colonies form a characteristic fried egg appearance, with a slightly raised centre and a mild spreading, irregular margin (Fig. 16.3a). Colonies do not adhere to the medium surface in the similar manner that *F. columnare* colonies do. Suspect isolates of *F. psychrophilum* may be characterized and confirmed using biochemical and physiological reactions (Bullock, 1972; Koneman *et al.*, 1988; Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Holt *et al.*, 1994; Schmidtke and Carson, 1995; Bernardet *et al.*, 1996; Cipriano *et al.*, 1996; Lorenzen *et al.*, 1997; Murray *et al.*, 1999; MacFaddin, 2000). Lorenzen *et al.* (1997) demonstrated phenotypic homogeneity of *F. psychrophilum* isolates from fish (primarily rainbow trout) experiencing clinical coldwater disease, with isolates from rainbow trout fry syndrome. They also showed that the concentration of the medium substrate could affect subsequent interpretation of biochemical testing results; for example, a substrate with too low concentration in the medium could produce a false negative. Furthermore, they emphasized the need to use fresh growth cultures as the inoculum for testing, and the use of sensitive test procedures for certain characters, such as lead acetate to detect weak production of hydrogen sulfide. Most *F. psychrophilum* isolates produce oxidase and catalase, hydrolyse gelatin and casein and produce flexirubin-like pigments (chromogenic shift in 10% KOH). Isolates typically do not grow, or grow poorly, on the rich nutrient concentration media routinely used in fish disease diagnostic laboratories, e.g. brain–heart infusion, tryptic soy and blood agar. Isolates are negative for starch, xanthine, chondroitin AC lyase and production of indole. Variable results are reported for tyrosine, elastin, nitrate reduction and assimilation of most sugars. Some of the variability in line-data results reported for certain tests could be attributed to differences in isolate origins; however, the methodologies utilized by various diagnosticians were likely a contributing factor. An example of a unique phenotype based on *F. psychrophilum* origins is illustrated with

certain Australian isolates, which produce brown pigment when grown on a medium containing tyrosine (Schmidtke and Carson, 1995).

F. columnare also grows poorly or not at all on high nutrient media. Cytophaga medium (Anacker and Ordal, 1959), the medium of Shieh (1980) or a selective bacteriological medium may be used (Bullock *et al.*, 1986; Hawke and Thune, 1992; Decostere *et al.*, 1997). Selective media increase the success for primary isolation of *F. columnare* by inhibiting the growth of contaminating environmental bacteria. Other formulated media for *F. columnare* generally consist of a base of tryptone and yeast extract supplemented with minimal salts and possibly a differential substrate, such as gelatin as in Hsu–Shotts medium (Bullock *et al.*, 1986). Song *et al.* (1988) evaluated various media for growth of *F. columnare*. Optimum incubation temperatures for *F. columnare* range from 25 to 30°C; however, many isolates will grow well at 37°C, and the diagnostician might use this criterion to aid with primary isolation by retarding the growth of environmental contaminants, many of which will not survive at 37°C. Colonies of *F. columnare* are small and often visible within 24 h, and are 3–4 mm in diameter after 48 h. Colonial growth of fresh isolates from tissues is characteristic and unique. The colonies are pale yellow and rhizoid, and adhere tightly to the surface of agar media (Fig. 16.3b). This characteristic colony growth, along with clinical signs, numerous long, thin cells in wet mounts or Gram-stained preparations, and case history comprise a presumptive diagnosis. Colonies should be transferred from primary plates to fresh broth or a semi-solid medium within 48 h to maintain their viability. The pale yellow colony pigment changes to pink when exposed to 3.0% potassium hydroxide. Confirmation of suspect *F. columnare* isolates can be accomplished with five phenotypic tests: growth in the presence of neomycin sulfate and polymyxin B, diffusible enzymatic degradation of gelatin and chondroitin sulfate A, and binding of aqueous Congo red dye adjacent to colonial growth on plates (Griffin, 1992). Various *F. columnare* isolates displayed uniformity in their production of extracellular proteases to degrade gelatin, casein, haemoglobin, fibrinogen and elastin (Bertolini and Rohovec, 1992). Molecular techniques are also used to confirm *F. columnare* (Bader *et al.*, 2003; Darwish *et al.*, 2004; Welker *et al.*, 2005; Panangala *et al.*, 2007).

Unique clinical signs are associated with BGD and offer experienced diagnosticians the opportunity for presumptive diagnosis by observing the fish. The disease signs include loss of appetite, gasping, lethargy, little to no response to external stimuli such as hitting the side of a tank or waving a hand over the fish, and swimming high in the water column in a characteristic 'soldier-like' head-first alignment towards and into the incoming water. These signs make it apparent that the fish are under high distress in their effort to respire. Numerous long, thin bacterial cells can be observed on gill tissues in wet mounts and by simple safranin staining or Gram-staining of gill tissue smears (Bullock, 1990). In wet mounts of excised gill tissues, severe hyperplasia of secondary lamellae will be obvious, along with numerous bacterial cells that are most evident along the periphery of affected tissues. Primary bacterial culture of *F. branchiophilum* from affected gills is not attempted routinely by fish health diagnosticians, because of the difficulty to culture and because of confidence with diagnosis based on the aforementioned criteria.

A number of immunological and molecular-based diagnostic methods have been reported for *F. psychrophilum*, *F. columnare* and *F. branchiophilum*. The immunofluorescent antibody technique (IFAT) has been used for the detection of *F. branchiophilum* and *F. columnare* from salmonid gills (Speare *et al.*, 1995), and Madetoja and Wiklund (2002) used IFAT in conjunction with nested polymerase chain reaction (PCR) to detect *F. psychrophilum* in the water from fish farms. More recently, a duplex IFAT method has been reported for the simultaneous detection of *F. columnare* and *E. ictaluri* in fish tissues (Panangala *et al.*, 2006). Enzyme-linked immunosorbent assay (ELISA) has been used to detect

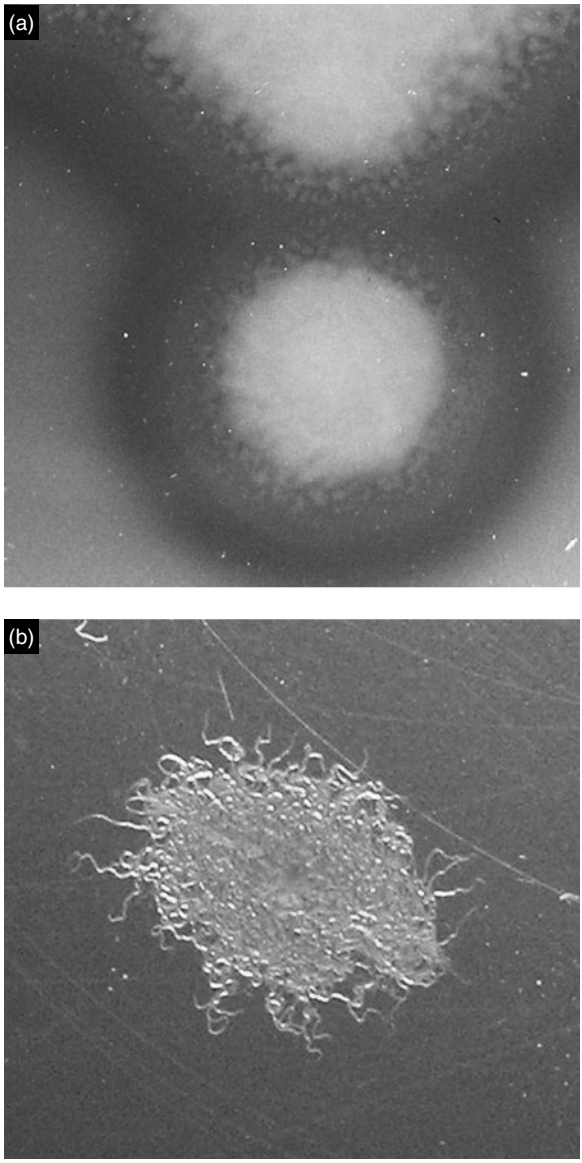


Fig. 16.3. Colonies of *Flavobacterium psychrophilum* (a) and *Flavobacterium columnare* (b). The bacteriological medium used in (a) was cytophaga agar (Anacker and Ordal, 1959) supplemented with 0.2% gelatin; the colonies were gelatinase positive, which is shown by the clear zones surrounding the colonies. The medium in (b) was cytophaga agar (Anacker and Ordal, 1959).

humoral antibodies in tilapia infected with *F. columnare* (Grabowski *et al.*, 2004). Polyclonal rabbit antibodies to *F. psychrophilum* lipopolysaccharide (LPS) O-polysaccharide (O-PS) have been used in ELISA to detect the pathogen in fish tissues, as well as to develop latex bead agglutination tests (Crump *et al.*, 2003). Serologic differences were observed among Danish origin *F. psychrophilum* isolates, compared with those from other European locations (Lorenzen and Olesen, 1997).

Serologic assays using antisera produced to specific isolates have been developed to detect *F. branchiophilum*. The IFAT has been used for both diagnostic and research purposes (Huh and Wakabayashi, 1987; Bullock *et al.*, 1994; Ostland *et al.*, 1994). Enzyme-linked immunoassays have been used to identify specific *F. branchiophilum* antigen

or host-produced antibody in response to *F. branchiophilum* (MacPhee *et al.*, 1985; Lumsden *et al.*, 1993). Immunodiffusion, immunoelectrophoresis and macroscopic slide and microtitre agglutination assays are additional serodiagnostic techniques that have been employed, primarily as research tools (Huh and Wakabayashi, 1989; Ostland *et al.*, 1989, 1994; Ko and Heo, 1997). Toyama *et al.* (1996) amplified (polymerase chain reaction) a segment of the 16s rDNA of *F. branchiophilum* and demonstrated specificity as the constructed DNA probes did not react with DNA from other related bacterial species including fish pathogenic *F. maritimus* and *F. columnare*.

Fish disease diagnosticians increasingly are utilizing DNA genotype-based assays to confirm the identity of fish pathogens. Numerous methods have been reported for the detection of Flavobacterial pathogens that use DNA amplification via PCR or other methods. Some of these assays were designed to target a single pathogen (Urdaci *et al.*, 1998; Cepeda and Santos, 2000; Wiklund *et al.*, 2000; Baliarda *et al.*, 2002; Bader *et al.*, 2003; Izumi *et al.*, 2005; Yeh *et al.*, 2006; Crumlish *et al.*, 2007), while others target several pathogens in a multiplex format (del Cerro *et al.*, 2002a; Panangala *et al.*, 2007; Altinok *et al.*, 2008). A quantitative PCR assay for *F. psychrophilum* has also been described (del Cerro *et al.*, 2002b).

Genome Structure and Transcription

The average genome size of bacterial species in the genus *Flavobacterium* estimated by DNA reassociation studies is 4.1±1 Mb (Fogel *et al.*, 1999). Genome sequencing has been completed for a virulent strain of *F. psychrophilum* (ATCC 49511; Duchaud *et al.*, 2007). Its circular chromosome is small (2,861,988 base pairs) compared with those of environmental members of the family, possibly a reflection of the organism's restricted niche. Another genome sequencing project is under way targeting *F. columnare* ATCC 49512. The *F. columnare* genome consists of a single circular chromosome with an estimated G+C content of 32% (Bernardet and Grimont, 1989), similar to that of *F. psychrophilum* (G+C content of 32.54%). To date, no genomic information is available for *F. branchiophilum*; however, the general characteristics of the chromosome are likely similar to *F. psychrophilum* and *F. columnare*.

Transcriptional information and possible gene product relationships with virulence come primarily from *F. psychrophilum* genome sequencing (Duchaud *et al.*, 2007). Proteases have long been thought to be essential virulence mechanisms for *F. psychrophilum* (Bertolini *et al.*, 1994), and indeed, 13 potentially secreted proteases have been found. Additionally, genes coding for cytolysins and haemolysin-like proteins rank high as virulence determinants. Fibronectin-type adhesins may play a role in the pathogen's ability to attach to the host, and a large repertoire of enzymes that combat oxidative stress probably aid in host defence during infection. A collagenase gene was identified, but was found to be interrupted by a mobile element (IS256) in rainbow trout strains. Thus, this secreted enzyme is probably not a necessary virulence factor. Many determinants have been found for the biosynthesis and export of extracellular polysaccharides that function in the production of biofilm. *F. psychrophilum* employs the breakdown of complex proteins and lipids to produce tricarboxylic acid cycle precursors, but is unable to use carbohydrates as carbon sources.

A chondroitin AC-lyase is considered to be important in the virulence of *F. columnare* (Xie *et al.*, 2005; Suomalainen *et al.*, 2006; Olivares-Fuster and Arias, 2008). Fish mucus has been shown to induce the production of several extracellular proteins and to alter the growth patterns of *F. columnare* (Staroscik and Nelson, 2008). These authors also demonstrated that fish mucus could support the growth of *F. columnare* as the sole carbon source. As with *F. psychrophilum*, adhesion and biofilm formation are important virulence determinants in *F. columnare*. Bader *et al.* (2005) demonstrated that *F. columnare* changed colony morphology, whole cell proteins, adhesion

and virulence when passed serially through media containing ampicillin. Virulence was unaffected when ampicillin-exposed isolates were injected, but was reduced in bath challenges.

Pathogenesis and Immunity

Reservoirs for *Flavobacterium* spp. pathogenic for fish are presumed to include asymptomatic, pathogen carrier fish and water supplies and sediment. Their primary mode of transmission is apparently horizontal and via infectious cells in the water column. Host stress and injury to the skin–mucus barrier greatly facilitate infections by *F. psychrophilum*, and ultimately coldwater disease (Madsen and Dalsgaard, 1999; Madetoja *et al.*, 2000). Moribund and dead fish with coldwater disease shed high numbers of viable *F. psychrophilum* cells into the tank water, and these serve as a supply for continuing infections (Madetoja *et al.*, 2000). Although *F. psychrophilum* may be transmitted horizontally, it is relatively difficult to produce mortality and clinical signs in laboratory-challenged fish in the absence of deliberate injury to the fish (Holt, 1987; Madsen and Dalsgaard, 1999; Madetoja *et al.*, 2000). Aoki *et al.* (2005) showed the importance of using logarithmic growth phase cultures for bath challenge; they immersed rainbow trout (averaging 1.3 or 5.6 g each) in cultures ≤24 h and fish developed clinical signs of rainbow trout fry syndrome.

Evidence suggests that vertical transmission is important for coldwater disease. *F. psychrophilum* has been recovered from ovarian fluid, egg surfaces, milt, mucus and kidney from mature coho and Chinook salmon and from rainbow and steelhead trout (Holt, 1987; Rangdale *et al.*, 1996; Brown *et al.*, 1997; Madsen *et al.*, 2005). Brown *et al.* (1997) recovered *F. psychrophilum* from the inside of both fertilized and eyed eggs. Ekman *et al.* (1999) isolated *F. psychrophilum* from both male and female sex products from (Baltic) Atlantic salmon (*S. salar*) returning from the Baltic Sea to spawn. The bacterium can also be transmitted to (the surface of) clean fish eggs through contamination in water containing *F. psychrophilum* cells, a form of horizontal transmission (Brown *et al.*, 1997; Rangdale *et al.*, 1997b; Kumagai *et al.*, 1998, 2000). Kumagai *et al.* (2000) exposed *F. psychrophilum* to eggs before or after water hardening, and eyed eggs. The eggs were then disinfected with 50 mg/l povidone-iodine for 15 min. The bacterium was recovered subsequently from those eggs only that were exposed to the pathogen prior to water hardening. This study also illustrated the importance of water hardening eggs in pathogen-free water. Evidence for internalization of *F. psychrophilum* in eggs was reported by Kumagai *et al.* (1998). They showed that disinfection with 50 mg/l povidone-iodine for 15 min was not effective to eliminate the pathogen in fertilized or eyed eggs that were contaminated prior to water hardening.

In addition to listlessness and loss of appetite, another early clinical sign of coldwater disease is erosion of fin tips. Bacterial colonization may appear in some fish as a whitish area on the fins, which can lead to separation of the fin rays. With chronic coldwater disease, the skin pigmentation in the peduncle area may also darken. In extreme cases of chronic coldwater disease, necrosis of the caudal region may be severe (Fig. 16.4) and progress until caudal vertebrae are exposed. With many fish, concurrent with or subsequent to external pathology is the establishment of an internal or systemic infection. Because abrasions to the skin–mucus barrier facilitate *F. psychrophilum* infections under laboratory exposures, presumably, abrasions aid with internalization of the pathogen in natural infections.

Innate immunity in rainbow trout to *F. psychrophilum* is related to spleen size (Hadidi *et al.*, 2008). These authors screened 71 full-sibling crosses and found that the resistant or susceptible phenotype was stable as size increased more than 300-fold. The spleen somatic index of 103 fish created high, medium and low spleen index groups. Animals with larger spleen indices were significantly more resistant to

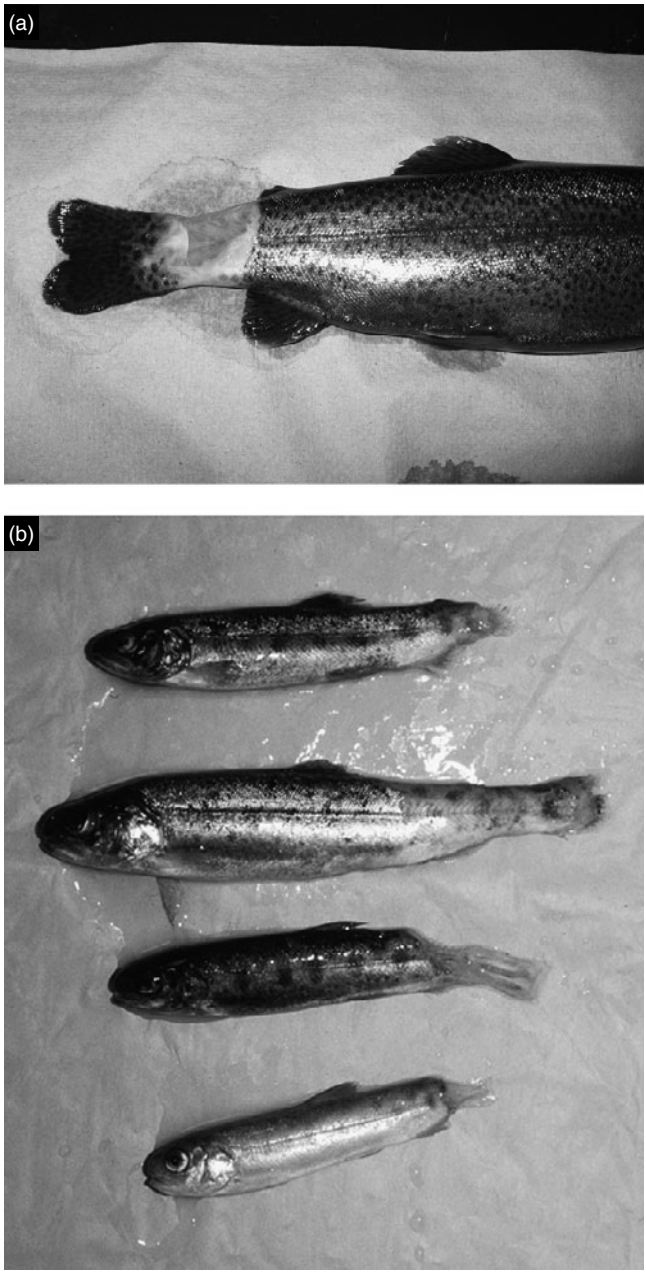


Fig. 16.4. Coldwater disease in rainbow trout (*Oncorhynchus mykiss*) (a) and coho salmon (b). Caudal lesions caused by chronic infections with *Flavobacterium psychrophilum*. Photographs courtesy of Vermont Fish and Wildlife Department, Waterbury, Vermont, and Wisconsin Department of Natural Resources, Madison, Wisconsin.

F. psychrophilum. Acute serum amyloid A (A-SAA) is normally thought to be a major acute-phase reactant and effector of innate immunity in vertebrates. When challenged with either *F. psychrophilum*, lipopolysaccharides (LPS), or CpG oligonucleotides, A-SAA was induced strongly in many immune-relevant rainbow trout tissues (Villarroel *et al.*, 2008). Unlike mammalian A-SAA, trout A-SAA does not increase in the plasma of diseased fish, which suggests that the role of this molecule in protection against *F. psychrophilum* (and other bacterial pathogens) is more likely to be involved in localized defence mechanisms.

Protective immune responses in rainbow trout have been shown to attenuated *F. psychrophilum* cultures (Álvarez *et al.*, 2008; LaFrentz *et al.*, 2008) and to a surface antigen P18 (Dumetz *et al.*, 2006). Formalin- and heat-inactivated *F. psychrophilum* induced protection in rainbow trout to subsequent intramuscular challenges (Madetoja *et al.*, 2006). Protective immunity to *F. psychrophilum* has also been demonstrated by vaccination of fish with an outer membrane fraction (Rahman *et al.*, 2002), as well as with a 70–100 kDa fraction (LaFrentz *et al.*, 2004) shown to be composed of O-polysaccharide components of LPS. Aoki *et al.* (2007) found that membrane vesicles (MVs) were released by *F. psychrophilum* into the growth medium at stationary phase. Stationary phase cells or MVs alone resulted in no protection, but survival to challenge was 94–100% when these two components were combined in experimental vaccines. Analysis of virulent and avirulent strains of *F. psychrophilum* by comparative immunoproteomic methods (Sudheesh *et al.*, 2007) found eight proteins unique to the virulent strain. Two highly immunogenic heat shock proteins, HSP 60 and HSP 70, share extensive homology with heat shock proteins of related bacteria. Passive immunizations also afford protection in rainbow trout using serum from convalescent and previously immunized fish (LaFrentz *et al.*, 2003).

Columnaris disease is most common in warm temperature waters, above 25°C. In pond culture, for example, as the water temperature increases, and if fish become stressed from crowding, reduced dissolved oxygen, handling or other factors, *F. columnare* may initiate disease (Wakabayashi, 1991). Skin abrasions or other injuries to the body surface may facilitate bacterial attachment and colonization by *F. columnare*. *F. columnare* contain surface components, including mucopolysaccharide and galactosamine, and these are important for initial attachment and for establishing an infection on the body surface (Johnson and Chilton, 1966; Thune *et al.*, 1993). In addition to the stress afforded to the host by poor quality water, Decostere *et al.* (1999) showed the presence of nitrite or organic matter increased *F. columnare* adhesion to gills of common carp (*C. carpio*). Extracellular proteases, particularly chondroitin lyase, are involved with production of necrotic tissue (Griffin, 1991; Bertolini and Rohovec, 1992). Bacterial proteases contribute to invasiveness and tissue damage and necrosis (Dalsgaard, 1993; Bertolini *et al.*, 1994; Nomura and O'Hara, 1994). External body surface lesions begin as small foci, often on the back and sides in the dorsal to caudal fin region. In the early stages, pale yellowish areas may be noted at the outer (i.e. leading) edges of lesions and at the foci of bacterial growth. As these lesions increase in size, often downward and along the sides of the fish, the epithelium may darken in colour. Necrosis of the epithelium and adjoining musculature leads to characteristic 'saddleback' lesions, because of their location adjacent to the dorsal fin. As the ulcer deepens, a bacteraemia may occur. However, systemic infections do not necessarily occur in all diseased fish (Hawke and Thune, 1992). Also, body surface lesions are not requisite for systemic infections. The gill tissue is another primary site of infection, starting on the tips of the lamella and progressing inward, with necrosis of the epithelium (Fig. 16.5). Body surface lesions are a cause for osmoregulatory imbalance for the fish, and the gill pathology and necrosis obviously impede respiration, with both factors contributing to potentially high morbidity and mortality.

Humoral antibody to *F. columnare* has been detected in salmonid fish, indicating previous pathogen exposure (Bullock,



Fig. 16.5. Gill necrosis in pumpkinseed (*Lepomis gibbosus*) (a) and white crappie (*Pomoxis annularis*) (b) affected by columnaris disease, *Flavobacterium columnare*. Photographs courtesy of Vermont Fish and Wildlife Department, Waterbury, Vermont.

1972). Humoral antibody titres were also shown in coho salmon that were vaccinated orally with heat-killed cells, and in rainbow trout exposed to a natural challenge with viable *F. columnare* (Fujihara and Nakatani, 1971). Mortality to the exposed rainbow trout was 52.0%, but the survivors were resistant to a subsequent exposure to the

pathogen. After a natural exposure to disease about 1 year later, the fish became immune carriers of the pathogen and had agglutinating antibody titres of up to 1:640 (Fujihara and Nakatani, 1971). Channel catfish (*I. punctatus*) produced agglutinating antibodies to *F. columnare* following subcutaneous and intramuscular injection with a heat-inactivated antigen (Schachte and Mora, 1973). Groups of channel catfish (1.5–4.1 g each) were exposed to formalin-inactivated *F. columnare* bacterins by immersion, each year, for 5 consecutive years. Vaccinated catfish showed less mortality and required fewer treatments for columnaris disease than did non-vaccinated controls (Moore *et al.*, 1990).

BGD outbreaks are often noted with predisposing factors that foster disease progression and severity. These factors include overcrowding or exceeding recommended rearing densities, low available dissolved oxygen, which can occur as a combination of crowding and reduced flow, and elevated un-ionized ammonia (NH_3). Increased turbidity in the water is also a contributing factor to disease outbreaks. At some facilities, heavy rainfall disturbs fine particle-size sediment in the water supply pipes or a spring water collection basin. When this sediment enters holding tanks, BGD outbreaks frequently have been noted to occur within a day or two. These sediment-related outbreaks have also been noted at facilities with closed water supplies (no feral fish in the water supply), and among apparently healthy fish and with no obvious stressors. The particulate material in the water likely irritates the gills, while the sediment may serve as a reservoir for *F. branchiophilum*. A significant correlation of BGD mortality with water supplies in hatch houses has been noted (Bebak *et al.*, 1997).

Seasonality appears to be a factor influencing BGD, as most outbreaks occur during the spring months. This coincides with not only increased water temperatures but also when hatcheries typically have their greatest numbers of fry and fingerling fish, which are more vulnerable to many diseases than adult fish. Bacterial attachment to and colonization of trout gill tissues are known to occur rapidly, usually within 1 h following waterborne exposure to *F. branchiophilum*, and within hours through cohabitation with infected fish (Ferguson *et al.*, 1991; Ostland *et al.*, 1995). Waterborne exposure is dose-dependent and although virulent and avirulent *F. branchiophilum* isolates adhere to gill tissues, only the virulent isolates are capable of colonization (Ostland *et al.*, 1995). Bullock *et al.* (1996) noted that ozone treatment of water in a fluidized sand recirculation system prevented BGD outbreaks in rainbow trout. However, the ozone treatments did not prevent *F. branchiophilum* from adhering to and colonizing gills. It was thought that epizootics were prevented, at least in part, because of the improved quality of the water by the ozone treatment.

Bullock (1972) induced a spontaneous BGD outbreak in stressed laboratory fish maintained in overcrowded conditions and with low dissolved oxygen and high ammonia. The causative bacterium was thought to be present in the spring water supply, which had no resident fish. Bullock (1972) was not successful in initiating an infection among stressed or control, non-stressed laboratory-maintained trout that were exposed to other bacteria from this spring water. Furthermore, Bullock (1972) could not induce BGD through cohabitation of healthy fish with fish experiencing natural disease. These results contrast with observations by others who have induced BGD successfully by immersion (Kimura *et al.*, 1978; Wakabayashi *et al.*, 1980; Kudo and Kimura, 1983c; Ferguson *et al.*, 1991). Furthermore, Ferguson *et al.* (1991) demonstrated transmission of BGD, through cohabitation, from diseased fish to various species and ages of healthy fish, including fish that were maintained in apparently optimal conditions, and in the absence of host predisposing stressors. MacPhee *et al.* (1995) showed that feeding fish just after a waterborne exposure to *F. branchiophilum* resulted in clinical signs and mortality, whereas unfed fish developed only mild clinical disease. They suggested the physiological effect of feeding facilitated colonization of *F. branchiophilum* to gill tissue. Wakabayashi *et al.* (1980) observed mortality in juvenile rainbow trout

that were exposed to laboratory cultures and concluded that one or more host predisposing stressor is required for experimental induction of BGD.

F. branchiophilum possess pili, a 23 kDa protein important for attachment to gills (Ototake and Wakabayashi, 1985; Heo *et al.*, 1990b). Fimbria was not detected on *F. branchiophilum* on rainbow trout gills in natural outbreaks of BGD, but glycocalyx, which might also facilitate adhesion, was detected (Speare *et al.*, 1991a). Ototake and Wakabayashi (1985) isolated the extracellular products from an *F. branchiophilum* growth culture and demonstrated both haemagglutinating and bacterioagglutinating activities and protease, phosphatase and phosphoamidase activity, but no haemolysin or endotoxin. They further demonstrated the importance of extracellular products *in vivo*. Washing *F. branchiophilum* cells to remove the extracellular products mechanically reduced infectivity to gill tissues dramatically. Following a waterborne challenge, no washed cells were detected on the gills of rainbow trout just 24 h after exposure. In contrast, there were 8.4×10^6 cfu unwashed cells/g of gill tissue 3 days after exposure. Juvenile rainbow trout immersed in cell-free extracellular products developed clubbing of gill filaments, followed by fusing of lamellae, and fish exposed to higher doses died. Kudo and Kimura (1983b) exposed fingerling rainbow trout to an *F. branchiophilum* bacterial cell extract, which induced fusion of secondary lamellae and filaments, hypertrophy and hyperplasia. They noted that the experimentally induced lesions were pathologically similar to those of fish from a natural infection with *F. branchiophilum* bacterial gill disease, but were less advanced than previously noted following waterborne challenge (Kudo and Kimura, 1983a).

Speare *et al.* (1991b) detailed the ultrastructural relation of *F. branchiophilum* to rainbow trout gill tissues prior to and during natural BGD outbreaks. Colonization by the bacterium was preceded by degeneration of microridges of the filament epithelium and slight alterations of the filament tips. Bacteria then spread to the proximal regions of filaments and lamellae. As colonization progressed, clinical signs became evident and death occurred to the host.

Wakabayashi and Iwado (1985) showed an increased lactate/pyruvate ratio in muscle tissue of rainbow trout exposed to *F. branchiophilum* by waterborne challenge. Muscle lactate levels were lower than those of uninfected control fish. They concluded that this was the result of impeded gas exchange at the gills, which affected the fish's ability to acquire oxygen and led to increased anaerobic metabolism and muscle lactate. Byrne *et al.* (1991) concluded that a decrease in muscle lactate indicated that hypoxia was not a primary cause of death to fish, but rather a contributing factor to the primary cause of death. They proposed a model that bacteria caused damage to respiratory cell membranes that resulted in the loss of osmotically active serum Na^+ and Cl–, and this caused fluid shifts from extracellular to intracellular compartments. Serum proteins and packed cell volumes increase, which results in haemoconcentrated blood. This circulatory disturbance is a primary cause of death, which is exacerbated by hypoxia. The apparent greater severity of BGD to young fish reflects their smaller ionic reserves (Byrne *et al.*, 1991).

Lumsden *et al.* (1993, 1995) demonstrated gill-associated antibody response in rainbow and brook trout. Lumsden *et al.* (1993) exposed 2-year-old brook trout to *F. branchiophilum* by cohabitation with infected rainbow trout. After 200 days, the surviving fish were immersed in 1.0×10^6 cfu viable *F. branchiophilum*. A response in both serum- and gill-associated antibody to *F. branchiophilum* was detected following both the primary and secondary exposures. The gill antibody response was significant, with a maximum titre of 1:16 on day 257, or 57 days following the second exposure. Lumsden *et al.* (1995) exposed rainbow trout (about 50 g each) twice, 6 weeks apart, to acetone-killed *F. branchiophilum* cells by bath or intraperitoneal injection. Gill and serum antibody responses were detected. Gill antibody responses to both challenge methods were greater and for longer duration following the second exposures. Fish

that were immersed in the highest concentration of antigen were also afforded the greatest protection. However, protection was not associated with a reduction in the number of bacteria on the gill surfaces. Heo *et al.* (1990a) exposed rainbow trout fingerlings to 4.0×10^7 cfu *F. branchiophilum*/ml by waterborne challenge, and followed this with a 2 min dip in 5% NaCl. This was done three times and at 6-day intervals. No antibody response to *F. branchiophilum* was detected in serum and they did not detect serum antibody in fish that had survived several natural infections.

Since BGD is an external disease, use of chemical therapy might impart a detrimental effect on gill-associated or mucosal antibody-producing cells. BGD epizootics are relatively short in duration and are severe. The onset of disease typically is quite rapid and if left untreated, high mortality may occur within a few days after disease signs are first noted. Young fish, which might have immature immune response capability, are most often affected.

Control, Treatment and Epizootiology

Intervention with antimicrobial therapy early in the disease process is advantageous to a successful treatment for coldwater and columnaris disease. Prophylactic or unjustified antimicrobial therapy should be avoided to minimize the development of antimicrobial resistance in fish pathogens (Rangdale *et al.*, 1997a; Bruun *et al.*, 2000; Schmidt *et al.*, 2000). Often, fish that are affected by bacterial diseases will lose appetite, which reduces treatment dosage and effectiveness of medicated food. Prior to the use of an antimicrobial to control mortality in fish, it is desirable to recover the causative bacterium, confirm the identification and perform *in vitro* sensitivity testing to ensure that the particular bacterial isolate is susceptible to the drug to be used. If a particular isolate happens to be resistant to the antimicrobial agent, the therapy will be of no benefit to the fish. If a treatment is administered for a resistant isolate, the likely results will include a financial loss for the medicated food and perpetuation of the resistant isolate at the facility.

Good techniques in fish husbandry will alleviate host stressors and minimize the risk, or prevent the introduction of pathogens to fish at rearing facilities and to wild populations through practices such as fish stocking (Wedemeyer, 2001). Disease preventive techniques include maintaining safe carrying capacities, quality and care of food, cleanliness of fish holding tanks and sanitization of equipment and placement of foot baths, etc. Proper feeding is especially important for columnaris disease; starved channel catfish showed reduced antibody titre response to *F. columnare*, and there was a significant increase in mortality relative to fish that were fed (Klesius *et al.*, 1999). Periodic health and pathogen inspections of fish lots would detect a pathogen prior to the expression of clinical disease. Affected fish (and the pathogen) might be confined within an area of the facility and a containment and treatment strategy could be implemented. Caution should be exercised when moving fish between hatcheries, especially if fish are suspected to be diseased or are known to be asymptomatic carriers. In the event of a disease outbreak at a rearing facility, it is important to remove dead fish promptly to reduce the source of infection to other fish (Madetoja *et al.*, 2000).

Various chemicals and antimicrobials have been used to treat *Flavobacterium* spp. in fish. Drugs to be used in fish destined for human consumption have been approved by the Food and Drug Administration in the USA for specific usage as indicated on the product's labels. Factors to be considered in order to maximize the effectiveness of a treatment include tissue clearance time, toxicity to various water chemistries and the organic load in the water.

Terramycin® (oxytetracycline) has been used to control coldwater disease mortality, especially in conjunction with improved environmental parameters to reduce stressors to fish. Antimicrobial susceptibility testing of *F. psychrophilum* isolates has been examined in an effort to provide fish

culturists with additional therapeutic agents (Rangdale *et al.*, 1997a; Bruun *et al.*, 2000). Early intervention to treat external coldwater disease has also been beneficial using potassium permanganate or a quaternary ammonium compound.

Treatment for columnaris disease is accomplished using chemicals or antimicrobial agents. An advantage to applying a chemical to the water is that it reduces the numbers of viable bacteria in the water column, thereby reducing re-infection. Hydrogen peroxide (35% PEROX-AID®) is used to control mortality in coolwater finfish and channel catfish; fry are treated at 50 mg/l (adults: 50–75 mg/l) for 1 h, for three treatments on alternate days (US Food and Drug Administration; http://www.fda.gov/cvm/drugsapprovedaqua.htm). Roccal® (Roccal®-D) and Hyamine, which are quaternary ammonium compounds, are used frequently to disinfect tanks and equipment for handling fish. Roccal® has also been effective at a concentration of 2 mg/l to treat BGD (Piper *et al.*, 1982); however, toxicity to fish may vary with water chemistry and the chemical lot, so care must be exercised when treating fish. Copper sulfate and potassium permanganate are two other chemicals that are effective, but water chemistry affects toxicity and efficacy (Jee and Plumb, 1981). A small-scale treatment on a few fish should be considered to evaluate toxicity prior to full-scale use. Aquaflor®-CA1 (florfenicol) is currently conditionally approved by the Food and Drug Administration (http://www.fda.gov/cvm/drugsapprovedaqua.htm) to control mortality in catfish due to columnaris disease. Aquaflor®-CA1 is delivered via medicated food and must be prescribed by a licensed veterinarian. Oxolinic acid has been administered as a dip or bath exposure at 10–12 mg/l on a smaller scale for external body surface cleansing. Terramycin® (oxytetracycline) is effective for systemic infections and is delivered via medicated feed at 55–82 mg/kg fish/day for 10 consecutive days (US Food and Drug Administration; http://www.fda.gov/cvm/drugsapprovedaqua.htm). Water in the range of pH 6.0 and soft water (i.e. 10 mg/l calcium carbonate $CaCO_3$ or less) are not conducive to persistence of *F. columnare* in the water column (Fijan, 1968).

Adjusting the water temperature is a simple technique that might aid with avoiding or controlling columnaris disease (Rucker *et al.*, 1953; Wood, 1974). An example of how this could be used was a diagnostic case that involved rainbow trout that were affected with columnaris disease and were being reared at a net-pen facility. The facility was situated below a dam and the culturists had the capability to adjust valves and acquire water from various sites high or low on the dam. The water temperature at the pens during the disease outbreak was about 18°C. The water supply valves were adjusted to increase the water flow from the lower part of the dam, which lowered the water temperature to about 13°C. Within a few days, the disease was controlled and the mortality ceased, and without the need for a chemical or antimicrobial treatment (C.E. Starliper, personal observation).

A vaccine (AQUA-COL; Intervet Inc., Millsboro, Delaware, USA) is available for catfish to provide protection from columnaris disease and mortality caused by virulent, wild-type isolates of *F. columnare*. Shoemaker *et al.* (2007) immersion-vaccinated eyed channel catfish eggs in a modified live *F. columnare* vaccine, or a 1:1 mixture (bivalent) of the modified live *F. columnare* vaccine and AQUAVAC-ESC™, which is a vaccine for protection from *E. ictaluri*. Both vaccines afforded some protection against subsequent bacterial challenge exposures to virulent *F. columnare*. Currently, there are no vaccines commercially available to protect fish against bacterial coldwater disease or bacterial gill disease.

A successful treatment of fish with BGD depends on early intervention. Total mortality will be significantly lower if a treatment is administered soon after the initial disease signs become evident. During active BGD and treatments, it is imperative to clean the raceways or tanks to minimize floating debris and reduce turbidity, which would be an additional irritant to gills. Affected

fish typically will not take food; therefore, food should be withheld during this time to reduce the severity of the outbreak. BGD was more severe in rainbow trout that were fed, and there was greater mortality compared with groups of fish that were not fed (MacPhee *et al.*, 1995). Hydrogen peroxide (35% PEROX-AID®) is approved to treat BGD in salmonids caused by *F. branchiophilum*. Treatment is at 100 mg/l for 30 min or 50–100 mg/l for 1 h; three treatments are given on alternate days (US Food and Drug Administration; http://www.fda.gov/cvm/drugsapprovedaqua.htm). Roberts (1994) used hydrogen peroxide to treat cutthroat and rainbow trout and recommended 50 mg/l for a 30 min bath, or 100 mg/l as a flow-through treatment for 1 h to fish smaller than 110/kg. Lumsden *et al.* (1998) infected rainbow trout (12–20 g each) with *F. branchiophilum* by immersion and subsequently demonstrated the effectiveness of hydrogen peroxide at various concentrations and treatment intervals.

Chloramine-T (sodium para-toluenesulfonchloramide) may be administered as a flow-through treatment at 6–15 mg/l for 1 h (From, 1980; Bullock *et al.*, 1991; Bowker and Erdahl, 1998). Multiple treatments may be necessary if the initial treatment has been administered well into the progression of the disease (Bowker and Erdahl, 1998). Benzalkonium chlorides have also been used to treat BGD (Piper *et al.*, 1982) at 1–2 mg/l active ingredient for 1 h, delivered either as a static bath or flow-through treatment. Commonly used forms of these compounds are Roccal (10% or 50% active) and Hyamine 1622 or 3500. Heo *et al.* (1990a) treated BGD successfully in rainbow trout fingerlings by immersion in a 5% NaCl bath for 2 min. Kudo and Kimura (1983c) showed that a treatment of rainbow trout fingerlings with 5% NaCl for 1 min was very effective to remove the bacterial pathogen and facilitate recovery.

Conclusions and Recommendations for Future Studies

Diseases and mortality to fish caused by *Flavobacterium* spp. affect nearly all cultured fish, and many free-ranging species. Poor fish husbandry and environmental parameters play significant roles in infections by Flavobacterial diseases, and on their severity. Similarly, maintenance of optimal rearing conditions reduces the risks for these diseases. *F. branchiophilum* is sometimes referred to as 'ubiquitous', and reservoirs of infection are thought to be the water supply and sediment and pathogen carrier fish. Specific reservoirs of infection are not well studied. Elucidation of the reservoirs, and their relationship with host stressors and environmental factors, could identify methods to prevent disease. Although many workers agree on the importance of host stressors to precipitate BGD, a specific parameter, or combination of parameters, has not been identified through laboratory studies. Studies to identify the host stressor(s) important for BGD could offer opportunities to predict disease outbreaks and, more importantly, prevent them from occurring.

Elucidation of virulence mechanisms has come primarily from the more easily cultured *F. columnare* and *F. psychrophilum*. Inspection of the complete genome sequence of *F. psychrophilum* has identified the potential of this organism to produce molecules known to be involved in attachment and invasion, and the same will be true for *F. columnare* when its genome sequence is completed. Transcriptome studies that compare the environmental state with the pathogenic state undoubtedly will highlight heretofore unknown virulence determinants, some of which may be targets for drug therapy or vaccines. Similar information from *F. branchiophilum* may have to await the application of whole genome and whole transcriptome techniques to single cells.

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17 Pasteurellosis and Other Bacterial Diseases

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Introduction

Bacteria that are well recognized as pathogens of fish have received a substantial amount of attention, as is evident from previous chapters. For other bacteria, there is less information available, either because the disease is rarely encountered or because it is a new and emerging problem. In other cases, it is not clear if the organism should be considered a pathogen or only an unusual concatenation of bacteria, host and environmental stresses.

This chapter focuses on five disparate groups of bacteria: *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*); the pseudomonads; members of the family Enterobacteriaceae other than *Yersinia* and *Edwardsiella; Francisella* and the atypical lactobacilli, including *Carnobacterium maltaromaticum;* and *Vagococcus salmoninarum*), which can be recognized as fish pathogens but about which there is not enough information to warrant separate chapters. Various other Gram-positive and negative bacteria that have been described recently as causing pathological conditions in fish are summarized briefly in Table 17.1.

A bias exists towards recognition of pathogens that affect those fish species reared in intensive aquaculture. Thus, the bacterial pathogens receiving individual chapter attention are pathogens of salmonid fish (e.g. aeromonads, vibrios, *Renibacterium*), channel catfish (*Ictalurus punctatus*) (e.g. *Edwardsiella* and *Aeromonas*) and yellowtail (*Seriola quinqueradiata*) (e.g. *Enterococcus*). Increased contact between fish and opportunity for transmission contribute to this prejudice. Stress due to crowding and culture conditions will also increase effects attributed to the pathogen. Not only are pathogens recognized more readily in intensive aquaculture, but this is also particularly the case in regions where there are diagnostic and pathology laboratories and fish health regulators, and where the costs of such infrastructure can be met. Warmwater aquaculture is probably under-represented in the list of significant pathogens. Factors that may result in a changed status for a bacterium could include new host species, more intensive and stressful conditions in aquaculture, exposure to new sources of potential pathogens and successful prevention of infection by a previously important species or strain of pathogen. In dealing with rare or emerging bacterial pathogens of well-studied systems and hosts, or in assessing the significance of pathogens in other hosts, it is important to designate the criteria

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Table 17.1. Fish bacterial pathogens for which there is limited information.

Bacterial species	Disease condition
Arcobacter cryaerophilus (*Campylobacter cryaerophila*) Aydin *et al*. (2000);Yildiz and Aydin (2006)	Infections of rainbow trout
Staphylococcus warneri Gil *et al*. (2000)	Mortalities in rainbow trout
Neochlamydia-like Draghi *et al*. (2007)	Epitheliocytis in Arctic char
Rhodococcus sp. Speare *et al*. (1991); Olsen *et al*. (2006a)	Granulomatous lesions in Atlantic salmon
Backman *et al*. (1990)	Panophthalmitis in Chinook salmon
Planococcus Austin *et al*. (1988); Austin and Stobie (1992a)	Gastroenteritis; slight swelling of the kidney
Janthinobacterium lividum Austin *et al*. (1992, 2003)	Mortalities in rainbow trout
Acidovorax delafieldii Aznar *et al*. (1992)	Eel (*Anguilla anguilla*) pathogen?
Unspeciated Gram-negative Ferguson *et al*. (1989)	Visceral necrosis of goldfish (*Carassius auratus*)
Acinetobacter sp. Roald and Hastein (1980)	Infection in Atlantic salmon
Moraxella sp. Baya *et al*. (1990a)	Mortalities in striped bass
Mycoplasma mobile Kirchhoff and Rosengarten (1984); Kirchhoff *et al*. (1987)	Gill isolate on tench (*Tinea tinea*) with red disease
Bacillus mycoides Goodwin *et al*. (1994)	Mortalities in channel catfish (*Ictalurus punctatus*)

Criteria for 'real pathogens':
1. Disease situation in fish, with significant mortalities (acute or persistent) or substantial damage or effect (e.g. growth rate, appearance, behaviour).
2. Problem recurs at different times and/or in different places (i.e. not a one-time event related to special stresses or environmental conditions. (A caveat here is that increasing awareness of the potential pathogenicity of a bacterium often leads to increased numbers of diagnoses and an apparent 'explosion' in occurrences of the disease.)
3. A recognizable agent can be isolated or detected from disease situations. Consistency of isolates? Virulent and avirulent strains?
4. Importance of predisposing stress and/or environmental factors for production of the disease.
5. Predominant isolate (or sole isolate) from organs, blood and/or lesions. A subclinical disease or a carrier state may be present when the same isolate occurs in many fish in a tested group. (Tissue samples taken aseptically from the organs of healthy fish show relatively low numbers of culturable organisms, even with enrichment steps.)

that are used to recognize a 'fish-pathogenic bacterium', rather than an isolation incidental to disease. How often are 'pathogens' isolated as a result of high loads of saprophytic bacteria (e.g. from sewage) meeting an unusually susceptible host or a host under unusually stressful conditions (e.g. *Proteus*)? What criteria should be used to determine the 'status' of a bacterium as a fish pathogen?

Photobacterium damselae subspecies *piscicida* (formerly *Pasteurella piscicida*)

Introduction

Pasteurellosis, also recently called pseudotuberculosis, was first observed in wild white

perch (*Morone americanus*) and striped bass (*M. saxatilis*) in 1963 at Chesapeake Bay, USA (Snieszko *et al.*, 1964; Allen and Pelczar, 1967). The causative bacterial agent for the disease was subsequently identified in 1968 and proposed to be under a new species, *Pasteurella piscicida* (Janssen and Surgalla, 1968).

Pasteurellosis has since been observed in cultured yellowtail (*S. quinqueradiata*) in Japan, with the causative agent confirmed as *P. piscicida* (Kubota *et al.*, 1970a,b; Kimura and Kitao, 1971; Kusuda and Yamaoka, 1972); in cultured gilthead sea bream (*Sparus aurata*) in Spain (Toranzo *et al.*, 1991); and in sea bream and sea bass in Europe (Magariños *et al.*, 1992a).

The biochemical and serological characteristics of *P. piscicida* isolates obtained from the USA, Japan and Europe were very similar. However, Gauthier *et al.* (1995) have reassigned the bacterium as *P. damselae* subspecies *piscicida*, following DNA–DNA relatedness of 80% between *P. piscicida* (NCIMB2058) and *P. damselae* subspecies *damselae* (ATCC33539). To date, the name *P. damselae* subsp. *piscicida* has been widely adopted.

In 1998, Thyssen *et al.* reported that the causative agent of pasteurellosis did not possess phenotypic evidence to be classified into a subspecies of *P. damselae*. The isolates from Japan, the USA and Europe differed in their pathogenicity, virulence factors, histopathological diagnosis and some biochemical characteristics. In this chapter, the interspecies differences among the isolates are presented and clarified.

The microorganism

P. damselae subsp. *piscicida* is a non-motile, Gram-negative rod bacterium having a size of 0.5×1.6 μm. It exhibits a bipolar staining, is oxidase- and catalase-positive, fermentative, does not produce gas from glucose and is halophilic (Janssen and Surgalla, 1968; Magariños *et al.*, 1992a).

P. damselae subsp. *piscicida* differs biochemically from the common fish pathogens *Vibrio anguillarum* and *A. salmonicida* subsp. *salmonicida*, as well as other *Pasteurella* spp. (Table 17.2). Compared with *Pasteurella* spp., *P. damselae* subsp. *piscicida* does not grow at 37°C, it is halophilic and does not produce nitrate. *P. damselae* subsp. *piscicida* is likewise different from *P. damselae* subsp. *damselae* in motility and biochemical characteristics (Table 17.3) (Thyssen *et al.*, 1998; Amagliani *et al.*, 2009).

P. damselae subsp. *piscicida* isolates from several European countries, Japan and the USA have been reported to be biochemically and antigenically similar, exhibiting the same homogeneous lipopolysaccharide (LPS) electrophoretic patterns and membrane protein profiles (Magariños *et al.*, 1992a) and fatty acid profiles (Romalde *et al.*, 1995c). Six Japanese isolates were found to have hydrophobic surfaces and agglutinated sheep red blood cells (Sakai *et al.*, 1993).

Molecular studies indicated that the majority of the European strains carried a 50 MDa plasmid (Magariños *et al.*, 1992a), while the Japanese strains possessed instead two plasmids with molecular sizes of 3.5 and 5.1 kb (Zhao and Aoki, 1992). Random amplified polymorphic DNA (RAPD)-PCR analysis also differentiated the European strains from the Japanese strains (Magariños *et al.*, 2000). The similarity between both groups based on Disc coefficient was estimated to be only 75–80%. From these, Magariños *et al.* (2000) assigned three types of *P. damselae* subsp. *piscicida* strains, namely the European strains (type 1), Japanese strains (type 2) and Israeli and other European strains (type 3).

The differences between the European and Japanese strains were further demonstrated when the molecular size of their sialic acid was determined to be 22 kDa and 26 kDa, respectively (Jung *et al.*, 2001b). Furthermore, the Japanese isolates were distinguished from European isolates at a cut-off value of 83% similarity in amplified fragment length polymorphism 16S rRNA gene sequences (Kvitt *et al.*, 2002).

On the other hand, Thyssen *et al.* (2000) observed that the G+C content of four strains of *P. damselae* subsp. *piscicida* (ATCC17911, 510/96, LA91-197 and NCIMB2058) were between 4.1 and 41.3%, while five strains (KUL22, ATCC35083,

Table 17.2. Characteristics of isolates of *Photobacterium damsela* subsp. *piscicida* (from Janssen and Surgalla, 1968; Yasunaga *et al.*, 1983, 1984; Toranzo *et al.*, 1991).

Gram stain	–	D-Tartrate	–
Bipolar staining	+	Gelatinase	–
Cell morphology	Short rods	Caseinase	–
Motility	–	Lipase (Tween 80)	+
Growth on nutrient agar	+	Phospholipase	+
In nutrient broth	+	Amylase	–
In peptone water	+	Haemolysis;	
On heart-infusion agar	+	Sheep erythrocytes	–
On BHI agar	+	Salmon erythrocytes	–
On SS agar	–	Acid production from:	
On MacConkey agar	–	Glucose	+
On Endo agar	–	Mannose	+
Growth at 5°C	–	Galactose	+
10°C	–	Fructose	+
15°C	+	Maltose	–
25°C	+	Sucrose	–
30°C	+	Rhamnose	–
37°C	–	Arabinose	–
Growth in 0% NaCl	–	Amygdaline	–
Growth in 0.5% NaCl	+	Melibiose	–
Growth in 3% NaCl	+	Mannitol	–
Growth in 5% NaCl	–	Inositol	–
Cytochrome oxidase	+	Sorbitol	–
Catalase	+	Glycerol	–
Methyl red	+	Xylose	–
Voges-Proskaur	+	Lactose	–
Indole production	–	Trehalose	–
Nitrate production	–	Raffinose	–
Ammonium production	–	Cellobiose	–
Citrate production	–	Dextrin	–
H_2S	–	Inulin	–
O/F	F	Glycogen	–
Gas from glucose	–	Adonitol	–
Arginine dihydrolase	+	Inositol	–
Lysine decarboxylase	–	Dulcitol	–
Ornithine decarboxylase	–	Eryhtritol	–
Tryptophan deaminase	–	Salicin	–
β-galactosidase (ONPG)	–	Aesculin	–
Urease	–		
Phenylalanine deamination	–		
Gluconate utilization	–		

H_2S, hydrogen sulfide; ONPG, σ-nitrophenyl β-D-galactopyranoside.

NCMB2184, ATCC33539 and 6–58) were between only 40.6 and 41.4%. Consequently, they analysed the strains using AFLP genomic fingerprinting and classified the species into five clusters; I (including *P. damselae* subsp. *piscicida* strains), II, IV, V and VI (including *P. damselae* subsp. *damselae*). Cluster I was subdivided further into two subclasses: Ia and Ib. Subclass Ia was composed of isolates from American strain ATCC17911 (white perch) and the Mediterranean Sea, whereas Subclass Ib included

Table 17.3. Characteristic differences between *Photobacterium damselae* subsp. *piscicida* and *damselae* (Smith *et al.*, 1991; Amagliani *et al.*, 2009).

Characteristic	*Photobacterium damselae* subspecies: *piscicida*	*damselae*
Motility	–	+
Nitrate reduction	–	+
Phospholipase	+	–
Urease	–	+/–
Esculinase	–	+
Chitinase	–	ND
Broth in 6% NaCl	–	ND
at 35°C	–	+
Uilization of :		
Pyruvate	–	+
Acetate	–	+
Production of acid from:		
Maltose	–	+
Cellobiose	–	+
Gas production	–	+/–
LCD	–	+/–
TCBS-1 growth	NG	GC
API 20E profile	2005004	6014004

ND, no data; NG, no growth; GC, green colonies; LCD, lysine decarboxylase production; TCBS, thiosulfate citrate bile sucrose agar plus 1.5% NaCl.

the American strain LA91-197 (striped bass) and all those originated from Japan.

Mannheim *et al.* (1978), however, argue that the classification of *P. piscicida* into the genus *Pasteurella* according to the capabilities of respiratory quinones and fumarate is not suitable. They believe that *P. piscicida* belongs to the family *Pasteurellaceae* based on rRNA cistron similarities (Ley *et al.*, 1990). The intergenic spacer region (ITS-2) of 16S and 5S rRNA, as well as the 16S and 23S rRNA intergenic spacer, was similar between *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida* (Osorio *et al.*, 2004a, 2005).

Geographical distribution and host range

P. damselae subsp. *piscicida* has been isolated from diseased fish in the USA (Janssen and Surgalla, 1968), Japan (Kimura and Kitao, 1971), Taiwan (Tung *et al.*, 1985) and, more recently, in Spain (Toranzo *et al.*, 1991), France and Greece (Baudin Laurencin *et al.*, 1991; Bakopoulos *et al.*, 1995), Italy (Ceschia *et al.*, 1991; Doimi *et al.*, 1991) and Portugal (Baptista *et al.*, 1996). The bacterium has been isolated from numerous species in both wild and farmed fish, as summarized in Table 17.4.

The disease

As mentioned, *P. damselae* subsp. *piscicida* causes in fish the disease known either as pseudotuberculosis or pasteurellosis. The disease is characterized by the presence of numerous white bacterial colonies throughout the internal viscera, particularly the spleen and kidney (Kubota *et al.*, 1970b). This disease is very serious in Japanese yellowtail culture, causing losses in individual farms of up to 50%. In Japan, the disease seems to occur in the early summer when water temperatures are 20–25°C. In a recent outbreak in Spain, water temperatures were also at 25°C and the outbreak occurred in the middle of the summer (Toranzo *et al.*, 1991).

This is one of the few fish pathogens that can cause massive mortalities in wild fish. Severe epizootics in white perch and striped bass populations occurred on the eastern seaboard of the USA in 1963 and 1977. Landings of white perch in the year after the 1963 epizootic were only 42% of those of any of the previous 3 years. The only significant cause for this decreased production appears to have been the *Pasteurella* epizootic in 1963 (Sindermann, 1990).

Little is known about the transmission of the disease. The bacterium, however, does not appear to survive in seawater for periods of greater than 3–5 days (Janssen and Surgalla, 1968; Toranzo *et al.*, 1982). This suggests that direct fish-to-fish contact is required for maintaining the infection in a population of fish. Magariños *et al.* (1994a) have reported that the bacterium may survive in seawater

Table 17.4. Isolations of *Photobacterium damselae* subsp. *piscicida* from fish.

Fish source		Citation
White perch	*Roccus americanus*	Snieszko *et al.* (1964)
Striped bass	*Morone saxatilis*	Snieszko *et al.* (1964)
Yellowtail	*Seriola quinqueradiata*	Kimura and Kitao (1971)
Black sea bream	*Mylio macrocephalus*	Muroga *et al.* (1977)
Japanese jack mackerel	*Trechurus japonicus*	Kusuda (1974)
Red sea bream	*Pagrus major*	Yasunaga *et al.* (1983)
Oval filefish	*Thamnaconus modestus*	Yasunaga *et al.* (1984)
Amberjack	*Seriola dumerili*	Kawahara *et al.* (1986)
Red spotted grouper	*Epinephelus akaara*	Ueki *et al.* (1990)
Ayu	*Plecoglossus altivelis*	Matsuoka *et al.* (1990)
Yatabe blenny	*Pictiblennius yatabei*	Hamaguchi *et al.* (1991)
Gilthead sea bream	*Sparus aurata*	Toranzo *et al.* (1991)
Turbot	*Scophthalmus maximus*	Toranzo *et al.* (1991)
Sole	*Solea solea*	Baudin Laurencin *et al.* (1991)
Mullet	*Mugil cephalus*	Baudin Laurencin *et al.* (1991)
Sea bass	*Dicentrachus labrax*	Baudin Laurencin *et al.* (1991)
Atherine	*Atherina boyeri*	Ceschia *et al.* (1991)
Sea bream	*Pagrus pagrus*	Ceschia *et al.* (1991)
Striped jack	*Pseudocaranx dentex*	Nakai *et al.* (1992)
Japanese flounder	*Parlichthys olivaceus*	Fukuda *et al.* (1996)
Largescale blackfish	*Girella punctata*	Kawakami *et al.* (1999)
Goldstriped amberjack	*Seriola aureovittata*	Kawakami *et al.* (2000)
Cobia	*Rachycentron canadum*	Lopez *et al.* (2002)
Hybrid striped bass	*M. saxatilis* × *M. chrysops*	Hawke *et al.* (2003)

in a 'viable but not culturable' state. Although these starved cells were not culturable by traditional methods, they were still virulent for turbot (*Psetta maxima*).

Obtaining primary clinical signs by experimental infection is very difficult. Wakabayashi *et al.* (1977) succeeded in infecting yellowtail artificially with a capsule containing cells of *P. damselae* subsp. *piscicida* yellowtail via the oral method. On the other hand, Kawahara *et al.* (1989) determined the fate of the invading *P. damselae* subsp. *piscicida* in yellowtail after infection by intramuscular injection, oral infection and immersion using histological and fluorescent antibody techniques. They argued that *P. damselae* subsp. *piscicida* either invaded the gills directly or through the gastrointestinal tract of yellowtail. Nagano *et al.* (2009), using a newly isolated strain from yellowtail and an immersion infection method, observed typical white spots on the spleen and kidney, and blood channels in the secondary gill filament of dead fish.

Likewise, maintaining virulence and pathogenicity of *P. damselae* subsp. *piscicida* is challenging. The longest preservation of the strain attained thus far is 6 months, using storage at –80°C in skimmed milk (Hashimoto *et al.*, 1985).

Diagnostic methods

Pasteurellosis can be detected by various methods: using gross pathologic internal signs such as granulomatous-like white spots in the kidney and spleen (Kubota *et al.*, 1970a; Toranzo *et al.*, 1987), by using the fluorescent antibody technique (Kitao and Kimura, 1974; Mori *et al.*, 1976), or by slide agglutination test using anti whole *P. damselae* subsp. *piscicida* cell sera.

Japanese strain

P. damselae subsp. *piscicida* strain from Japan can be detected rapidly (1 h) using the fluorescent antibody technique compared with the culture method (Kawahara *et al*., 1986). The strain can also be observed by direct hybridization, using species-specific DNA fragment probe cloned from the chromosomal DNA from *P. damselae* subsp. *piscicida*. For example, the number of pathogens from water and in fishes has been counted by colony hybridization (Zhao and Aoki, 1989). Recently, the strain has also been detected by modified ELISA, which offers a simple and semi-quantitative method (Jung *et al*., 2001a).

Zhao and Aoki (1992) found one large plasmid (110 kb) and two small plasmids (3.5 and 5.1 kb) from strains of *P. damselae* subsp. *piscicida* isolated from yellowtail in Japan. The 5.1 kb plasmid (pZP1) was a common plasmid found in all isolates. Using the pZP1-1a/1b primer set, a 484 bp DNA fragment was amplified from all Japanese and US strains from the kidney of naturally infected yellowtail (Aoki *et al*., 1997), suggesting that the primer set would be useful for rapid diagnosis of pasteurellosis in marine fish in Japan. Interestingly, however, pZP1 is not found in European strains (Magariños *et al*., 1992a: Osorio *et al*., 1999).

Molecular diversity can also be used to detect *P. damselae* subsp. *piscicida*; by pulsed-field gel electrophoresis (Kijima-Tanaka *et al*., 2007), by immunopolymerase chain reaction (Kakizaki *et al*., 1996) and by oligonucleotide DNA array (Matsuyama e*t al*., 2006).

European strain

P. damselae subsp. *piscicida* from Europe can be diagnosed rapidly by a latex agglutination test (BIONOR Mono-kit). The latex kit reacts with all strains of *P. damselae* subsp. *piscicida* with no cross-reactions with *V. anguillarum* and *P. multocida*, *P. haemolytica* and *Haemophilus parasuis* (Romalde *et al*., 1995a,b). Another method, the magnetic bead-EIA (enzyme immunoassay) could also detect the strain. However, this method reacted with *P. damselae* subsp. *damselae* and *P. histaminum* when the bacterial concentration was high (10^9 to 10^{10} bacteria/ml; Romalde *et al*., 1999). So far, the most sensitive and rapid method is the enzyme-linked immunosorbent assay (ELISA) developed by Bakopoulos *et al*. (1997b).

Genetic-based diagnosis has also been found to be effective in detecting *P. damselae* subsp. *piscicida*; Osorio *et al*. (1999) developed a PCR method using primers (Car 1 and 2) of 16S rRNA gene sequence; likewise, Osorio *et al*. (2000b) developed a colony hybridization method for diagnosis, making use of the presence of the *ureC* gene found in *P. damselae* subsp. *damselae* strains only. Rajan *et al*. (2003) used random amplification of polymorphic DNA (RAPD) genomic fingerprints, Valle *et al*. (2002) used the capsular polysaccharide gene, Gonzalez *et al*. (2004) employed thiosulfate citrate bile salts–sucrose agar and Amagliani *et al*. (2009) developed a multiplex polymerase chain reaction protocol.

Histopathological diagnosis

YELLOWTAIL IN JAPAN. Pasteurellosis usually occurs in cultured marine fish such as yellowtail and red sea bream, but also in wild fish. Diseased fish die in the bottom of net cages without visible external signs. Internally, however, they display white spotted lesions within visceral organs such as the spleen, kidney, liver and heart. These white spotted lesions vary in size and are markedly formed, especially in the spleen and kidney. Histopathological features of these white-spotted lesions are well studied in diseased yellowtail. Lesions are bacteria-colonial lesions and granulomatous lesions. The early stages of infectious lesions are comprised mainly of many intracellular bacterial colonies. Splenocytes and macrophages in the spleen, reticuloendothelial cells and macrophages in the haematopoietic tissues phagocytose invading bacteria and allow their intracytoplasmic propagation because of no/slight bactericidal activities. Enlarged lesions are comprised of many intracellular bacterial colonies,

free bacterial cells and necrotic tissues. Small-sized bacterial colonies are often surrounded by accumulated macrophages to form nodular lesions. These nodular lesions develop into granulomas in which epithelioid cell layers encapsulate bacterial colonies and tissues showing coagulation necrosis. Within old granulomas, bacteria are markedly reduced. Diseased fish usually die of functional failure due to multiple production of bacterial colonies and/or granulomatous lesions in visceral organs (Kubota *et al.*, 1970a,b, 1982).

SEA BASS, SEA BREAM AND SOLE IN EUROPE. Diseased fish show enlarged spleens with whitish lesions (Toranzo *et al.*, 1991; do Vale *et al.*, 2007b). Histopathologically, infected lesions are formed mainly in the spleen, kidney, liver and digestive tract. The lesions are observed as focal necrosis with extracellular bacterial colonies in the spleen and haematopoietic tissues in the kidney. In the lesions, tissue necrosis is marked and bacteria are propagated extracellularly with slight bacteria-phagocytosis by macrophages and neutrophils. The pathogen produces exotoxin to induce apoptosis of these inflammatory cells, so their bacteria-phagocytosis results in disturbance.

Pathogenesis in European strains

Invasion

The antigenic capsular layer is one of the important virulent factors of *P. damselae* subsp. *piscicida* from Europe (Magariños *et al.*, 1996a). Salati *et al.* (1989) described that the LPS of the pathogen contained 18–24% sugar and 34–36% fatty acids. The LPS showed acute toxicity of 30–81 mg/kg in mice. The capsular polysaccharide produced by the pathogen was also purified and characterized, and the polymer was composed of glucose/mannose/*N*-acetylgalactosamine/galacturonic acid/acetic acid in the molar ratios of approximately 2.5:1.3:0.5:0.4:2.5, respectively (Bonet *et al.*, 1994). The presence of a capsule confers resistance to serum killing and increases lethality to fish. Magariños *et al.* (1996a) suggested that the property of serum resistance of the capsule was associated closely with the persistence of the pathogen in its host. The capsule also plays an important role in the reduction of phagocytic activity by macrophages in sea bream (Arijo *et al.*, 1998). However, there are no significant differences in the invasiveness between the capsulated and non-capsulated strains (Lopez-Doriga *et al.*, 2000). *P. damselae* subsp. *piscicida* invasiveness was more efficient at low m.o.i., reaching saturation at higher m.o.i. (multiplicity of infection), suggesting that internalization might be receptor-mediated. Normal sea bass serum slightly reduced the invasion capability of the virulent strain (MT1415), but heat-inactivated normal serum had no effect. On the other hand, heat-inactivated fish antiserum raised against the virulent strain reduced the percentage of invading epithelial cells by 50%. As for other pathogens, an intracellular phase of *P. damselae* subsp. *piscicida* may be a mechanism to delay or avoid phagocytosis and host immune response, favouring the spread of infection (Lopez-Doriga *et al.*, 2000). Furthermore, it is capable of adhering, entering and surviving within the non-phagocytic epithelial cell line SAF-1 of gilthead sea bream (*S. aurata*), which may be important for persistence and establishment of a carrier state in the sea bream (Acosta *et al.*, 2009). Adherence and invasive capacities of European and Japanese strains have also been investigated. Magariños *et al.* (1996b) indicated that the pathogen exhibited high binding capacities to CHSE-214 cells or fish intestine. The binding capacities were affected by heat and sugars, but not by proteinase K or by treatment of antiserum. Japanese strains also adhered to CHSE-214 and EPC cells (Yoshida *et al.*, 1997). *P. damselae* subsp. *piscicida* have high hydrophobicity and lectin activities, suggesting that these characteristics could be associated with adhesion of the bacteria to host cells (Sakai *et al.*, 1993).

Macrophages

The *P. damselae* subsp. *piscicida* from Europe in sea bass (*Dicentrarchus labrax*)

induces apoptosis of macrophages and neutrophils simultaneously (do Vale *et al.*, 2003). The eventual cell-lysis in high numbers of the two phagocytic cells appears as a novel and very powerful pathogenic strategy of this pathogen. Do Vale *et al.* (2005) found a novel plasmid encoded with apoptosis-inducing protein of 56 kDa (AIP56) exotoxin secreted abundantly by all EU strains, although this plasmid was not found in strains isolated in Japan. Systemic macrophage and neutrophil destruction by secondary necrosis was induced by a bacterial exotoxin in a Gram-negative septicaemia (do Vale *et al.*, 2007a). Detachment-induced apoptosis of enterocytes was also observed in the gut (do Vale *et al.*, 2007b).

Noya *et al.* (1995a) indicated that the peritoneal exudate cells in the gilthead sea bream were capable of phagocytosing and killing *P. damselae* subsp. *piscicida*. However, the macrophages appeared to be unable to kill it. The host resistance (sensitivity) may be related to the efficiency of its phagocytes (Noya *et al.*, 1995a). After intraperitoneal injection, no phagocytosed pathogens were observed in the peritoneal eosinophilic granule cells (EGCs) at any time. However, abundant phagocytosed pathogens were observed in other types of granular leucocytes and macrophages of the peritoneal exudate (Noya and Lamas, 1997). In the hybrid striped sea bass, *P. damselae* subsp. *piscicida* is capable of surviving and replicating in the macrophage as an intracellular pathogen (Elkamel *et al.*, 2003). Thus, intracellular pathogens like *P. damselae* subsp. *piscicida* have evolved a variety of strategies to circumvent host cell mechanisms and which enable them to survive and replicate within potentially lethal phagocytic host cells (Elkamel *et al.*, 2003).

Extracellular products (ECP)

Extracellular products (ECP) from *P. damselae* subsp. *piscicida* strains, which were isolated from gilthead sea bream, rainbow trout (*Oncorhynchus mykiss*) and turbot, did not possess strict host specificity and were strongly toxic for these fish. The ECP extracts from strains isolated from Italy, France, Japan and the USA were cytotoxic for fish and homoisothermic cell lines. They possessed notable phospholipase activity and displayed haemolytic activity for sheep, salmon and turbot erythrocytes (but not for trout erythrocytes) (Magariños *et al.*, 1992b). The activity of ECP such as caseinase increased in bacteria cultured under iron-limited conditions (Bakopoulos *et al.*, 1997c). ECPs, bacterial cells and cell components isolated after being grown in selected media were compared in virulence–toxicity studies. Certain formulations based on yeast extract and bacterial and/or fish peptone promoted the toxicity of ECPs when *P. damselae* subsp. *piscicida* cells were administrated (Bakopoulos *et al.*, 2002). In sea bass, ECPs produced *in vivo* showed higher toxicity than the ECPs produced *in vitro*. Histologically, the inflammatory responses associated with the injection of the pathogen and its ECP were similarly effective (Noya *et al.*, 1995c). Histological examination of these ECPs revealed inflammatory and necrotic lesions in the spleen, liver, head, kidney, intestine and heart post-introduction of the ECPs (Bakopoulos *et al.*, 2004). The ECPs were not good immunogens in their soluble form, despite various treatments prior to immunization, although the crude capsular polysaccharide was an important immunogen (Bakopoulos *et al.*, 2003).

Nitric oxide (NO) and peroxynitrite

While nitric oxide (NO) alone is sufficient to cause damage to *P. damselae* subsp. *piscicida*, growth in glucose-supplemented medium enhances protection to reactive oxygen intermediates rather than NO (Acosta *et al.*, 2003). The capsulated strains are more resistant to the bactericidal action of NO and peroxynitrites than the non-capsulated strains. Blocking the NO response of the fish results in greater susceptibility to *P. damselae* subsp. *piscicida* (Acosta *et al.*, 2004).

Superoxide anion and superoxide dismutase (SOD)

The increased production of superoxide anion by rainbow trout macrophages infected with *P. damselae* subsp. *piscicida* coincides with the highest bactericidal activity. This suggests that the superoxide anion could be involved in the killing of the pathogen (Skarmeta *et al.*, 1995). The catalase produced by *P. damselae* subsp. *piscicida* was expressed constitutively, although its SOD appeared to be repressed under low oxygen conditions. In spite of the presence of a periplasmic SOD, *P. damselae* subsp. *piscicida* was susceptible to killing by exogenous superoxide anion generated in a cell-free system. Addition of exogenous SOD to this system did not abolish the bactericidal effect; however, the addition of catalase was protective. Thus, it is suggested that lack of periplasmic catalase is implicated in susceptibility to killing by reactive oxygen species (Barnes *et al.*, 1999). On the peroxidase activity, Diaz-Rosales *et al.* (2003) suggested that the resistance (survival) rate to hydrogen peroxide in the virulent strain was higher than that in the non-virulent strain. The presence of higher levels of catalase activity in *P. damselae* subsp. *piscicida* is probably concerned with its virulence (Diaz-Rosales *et al.*, 2003). Incubation of a virulent *P. damselae* subsp. *piscicida* with sole (*Solea senegalensis*) phagocytes has shown a higher survival rate than that of an avirulent strain. The increased levels of catalase activity detected in the virulent strain indicated a possible role for this enzyme in bacterial survival (Diaz-Rosales *et al.*, 2006).

Iron

Iron is very important for the pathogenesis of *P. damselae* subsp. *piscicida* strains isolated in the EU and Japan (Magariños *et al.*, 1994c). Expression of capsular polysaccharide is dependent on iron availability and growth phase (do Vale *et al.*, 2001). The dietary iron increases susceptibility of sea bass to the pathogen (Rodrigues and Pereira, 2004). Thus, pathogens must possess effective iron acquisition systems in order to sequester the iron bound to proteins. Outer membrane proteins of virulent *P. damselae* subsp. *piscicida* (EU) strains are capable of binding haemin, and its binding activity is increased by iron limitation. The mechanisms rely mainly on the direct interaction between the haemin molecules and surface-exposed outer membrane protein receptors (Magariños *et al.*, 1994c; do Vale *et al.*, 2002). Bacteria have developed a cascade of iron scavenging and transport systems. Iron homeostasis must be strictly controlled. Expression of many genes involved in iron uptake systems is regulated at the transcriptional level by the Fur (ferric uptake regulator) repressor protein (Escolar *et al.*, 1999). In *P. damselae* subsp. *piscicida* (EU) strains, the gene encoding the ferric uptake regulator (*fur*) protein was cloned, and the ORF of 477 bp encoded for a protein of 148 amino acids (Juiz-Rio *et al.*, 2004). Novel Fur-regulated genes coding a putative complete iron-regulated outer membrane transport protein and a transport protein (pseudogene) were also identified by the Fur titration assay (FURTA) to discover iron-regulated genes (Osorio *et al.*, 2004b). The haem uptake gene cluster including putative proteins (HutZ, HuX and HutW), three components of TonB system (TonB, ExbB and ExbD), the putative periplasmic binding protein (HutB), the putative inner membrane permeases (HutC), the putative ABC-transporter ATP-ase (HutD) and the outer membrane haem receptor (HutA), were identified in *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae* strains, although the *hutA* gene was absent in a *P. damselae* subsp. *piscicida* strain D121 or was interrupted in some strains constituting a pseudogene (Juiz-Rio *et al.*, 2005a,b).

One of the main strategies to obtain iron is the synthesis and secretion of Fe(III) chelators, named siderophores, which are low molecular mass iron-chelating molecules that can remove iron from host iron-binding proteins (Crosa, 1989). The differential occurrence of siderophore biosynthesis gene cluster from *P. damselae* subsp. *piscicida* was low compared with that of other *Vibrio* spp. This gene was

related structurally and functionally to the *Yersinia* high-pathogenicity island (Osorio *et al*., 2006). *P. damselae* subsp. *piscicida* can acquire iron from free haem or haem-containing proteins present in the host tissues by a siderophore-independent mechanism. The haem uptake consists of an outer membrane receptor that transports the haem molecule into the periplasm via a TonB-dependent process, and additional proteins that complete the transport of haem from the periplasm into the cell cytoplasm. Expression of haem uptake genes is iron-regulated at the transcriptional level by the repressor protein Fur. The haem uptake mechanisms are believed to contribute to virulence in fish (Lemos and Osorio, 2007).

Mobile elements

DNA fragments identified from the virulent *P. damselae* subsp. *piscicida* EU strains by subtractive hybridization included homologues of siderophore biosynthesis and homologues related to mobile elements. One of these was related to the *V. cholerae* SXT element (Juiz-Rio *et al*., 2005b), which was a representative of a family of conjugative transposon-like mobile genetic elements that encoded multiple antibiotics-resistance genes (Beaber *et al.*, 2002). The SXT elements are functionally and structurally similar to the conjugative element R391 on genomic islands, conjugative transposons and bacteriophages. Osorio *et al*. (2008) carried out genomic and functional analyses of integrating conjugative elements (ICEs) derived from the virulent *P. damselae* subsp. *piscicida* EU strain, PC554.2, and found that the elements were indeed related closely to the SXT and R391 elements and could transfer a virulence gene.

Plasmids have been isolated from both European and Japanese strains. Magariños *et al*. (1992a) reported that a plasmid of 53 kDa was present in the majority of European strains, while Zhao and Aoki (1992) isolated two plasmid DNAs, 3.5 and 5.1 kb. The larger 5.1 kb plasmid was common to all strains isolated in Japan.

Pathogenesis of Japanese strain

The cell surface hydrophobicity of *P. damselae* subsp. *piscicida* strain, which has been isolated in Japan, is high. The lectin activity of this pathogen was examined through haemagglutination with sheep and rainbow trout red blood cells. All strains showed lectin activities and several agglutinations were inhibited by sugar (Sakai *et al*., 1993). Naka *et al*. (2005c) cloned a haem receptor gene from *P. damselae* subsp. *piscicida*. The gene has an open reading frame (ORF) consisting of 2154 bp with a predicted 718 amino acid residue. *P. damselae* subsp. *piscicida* belongs to the haem receptor family. Its iron-binding system involves more than one receptor and its virulence is related to their iron-uptake system. Naka *et al*. (2005b) likewise cloned iron uptake-related genes *tonB1*, *tonB2* and the iron uptake regular or fur gene. Using knockout mutant, they showed that *tonB1* was regulated by fur, and fur influenced the expression of the haem receptor gene, *hut a*. A phospholipase gene has also been cloned from *P. damselae* subsp. *piscicida* (Naka *et al*., 2007). Its ORF consisted of 1218 bp encoding a protein of 405 amino acids. A recombinant protein of phospholipase showed lectin-dependent haemolytic activity, suggesting that phospholipase might play an important role during iron uptake in the host's erythrocytes. Naka *et al*. (2005a) performed random sequencing of the genomic DNA of *P. damselae* subsp. *piscicida* and they produced contigs containing 2055 ORFs that have homology with genes in the GenBank database. Some ORFs have homology with reported virulence-related genes, classified as encoding colonization factor (polar flagellar-related genes, capsule-related genes), accessory colonization factor (superoxide dismutase [Cu-Zn], MshA biogenesis protein and haem receptor genes), exotoxin-related genes (2 haemolysins, phospholipase, hyaluronidase and lysophospholipase L2 genes) and lipopolysaccharide-related genes.

Elkamel *et al*. (2003), using *in vitro* assay, evaluated the ability of *P. damselae*

subsp. *piscicida* to survive and replicate within macrophages from hybrid striped bass and concluded that it was an intracellular pathogen that could survive and multiply within macrophages.

Control and treatment

Chemotherapy

Many chemotherapeutic agents exhibit high antibacterial activity against *P. damselae* subsp. *piscicida*, including amoxicillin (Kitao *et al.*, 1989), ampicillin (Kusuda and Inoue, 1976, 1977), bicozamycin benzoic acid (Kitao *et al.*, 1992), florfenicol (Fukui *et al.*, 1987; Yasunaga and Tsukahara, 1988; Yasunaga and Yasumoto, 1988), flumequine (Takahashi *et al.*, 1990), novobiocin, oxolinic acid (Endo *et al.*, 1973; Takahashi and Endo, 1987), piromidic acid (Katae *et al.*, 1979), fosfomycin, sulfisozole and thiamphenicol (Aoki, 1992; Bakopoulos *et al.*, 1995; Kawanishi *et al.*, 2004; Martinez-Manzanares *et al.*, 2008) and oxytetracycline (Toranzo *et al.*, 1991; Hawke *et al.*, 2003). These chemotherapeutics have been widely used for treatment of pasteurellosis in marine fish (Perciformis) farms. For example, in Japan, treatment of yellowtail infected with *P. damselae* subsp. *piscicida* is permitted provided that the antimicrobial agents, dosages and withdrawal times of chemotherapeutics allowed by the Japanese Fisheries Agency are followed (Table 17.5). The recommended period for oral administration of each drug to fish is 5–7 days.

The culture supernatant (bacteriocin-like substance) of *Vibrio* sp. strain NM10 inhibited *P. damselae* subsp. *piscicida* (Sugita *et al.*, 1997), as well as vanicoside A and B, antimicrobial compound, which was isolated from *Polygonum sachalinense* rhizomes (Kumagai *et al.*, 2005).

P. damselae subsp. *piscicida* infection occurred frequently in cultured yellowtail. In Japan, control of the pathogen was started in 1979 using antimicrobial agents. However, multiple drug-resistant strains of *P. damselae* subsp. *piscicida*, which showed resistance to chloramphenicol (CM), furazolidone (NF), kanamycin (KM), sulfonamide (SU) and tetracycline (TC), appeared suddenly in yellowtail farms in 1980 (Aoki and Kitao, 1985). The infection with multiple drug-resistant strains has continued until today, excluding a certain period in the 1990s. Later, oxolinic acid, ampicillin (AMP) and florfenicol (FF) were also developed for the treatment of pasteurellosis (Takashima *et al.*, 1985; Kusuda *et al.*, 1988a, 1990; Kim *et al.*, 1993). However, drug-resistant strains likewise have appeared and increased in yellowtail farms. The resistance markers of *P. damselae* subsp. *piscicida* strains isolated from cultured yellowtail were a combination of AMP, CM, FF, KM,

Table 17.5. Chemotherapeutic agents approved for pseudotuberculosis of yellowtail in Japan.

Chemotherapeutic agents	Route of administration	Dosage	Withdrawal time
Amoxicillin	Oral	40 mg/kg	5 days
Ampicillin	Oral	20 mg/kg	5 days
Fosfomycin calcium	Oral	40 mg/kg	15 days
Florfenicol	Oral	10 mg/kg	5 days
Bicozamycin benzoic acid	Oral	10 mg/kg	27 days
Novobiocin	Oral	50 mg/kg	15 days
Oxolinic acid	Oral	30 mg/kg	16 days
Sulfisoxasole	Oral	100–200 mg/kg	10 days
Thiamphenicol	Oral	50 mg/kg	15 days

NA, NF, SU and/or TC. Transferable R plasmids were detected in these strains of *P. damselae* subsp. *piscicida*. The detected R plasmid encoded combinations of AMP, CM, FF, KM, SU, TC and/or trimethoprim (TMP). The sequence structures of some of the transferable R plasmids have been determined, including that of pP99-018 (150.057 bp) and pP91278 (131,529 bp) detected in Japan and the USA, respectively. The two plasmids were highly conserved, with differences due only to variation in the drug resistance and conjugative transfer regions. It is believed that these plasmids are related closely to the Inc J element, R391, a conjugative, self-transmitting, integrating element, or constin (Kim *et al.*, 2008).

The nucleotide sequences of drug-resistance genes that originate from human, domestic animal and fish-pathogenic bacteria have been registered in GenBank. The drug-resistant genes were classified based on their nucleotide sequences. The chloramphenicol-resistance genes were classified into 25 CAT types, and cat genes encoding R plasmids from *P. damselae* subsp. *piscicida* were classified into CAT I and II (Kim *et al.*, 1993; Morii *et al.*, 2003). Tetracycline resistance genes were classified into 32 types and tet genes from *P. damselae* subsp. *piscicida* were identified into tet (D) (Kim and Aoki, 1994). The kanamycin-resistant gene of *P. damselae* subsp. *piscicida* was classified into aphA7 type in 51 types of aminoglycoside-resistant genes (Kim and Aoki, 1994), pp-fro types in five types of forfenicol-resistant genes (Kim *et al.*, 1993; Kim and Aoki, 1996a), and Sul II type in three types of sulfonamide-resistant genes (Kim and Aoki, 1996b). The ampicillin resistance gene cloned from R plasmid of *P. damselae* subsp. *piscicida* was classified as a class A beta-lactamase (Morii *et al.*, 2004). A rapid method to detect an ampicillin-resistant strain using the iodometric method has been developed (Kitao and Aoki, 1983). This method can detect an ampicillin-resistant strain directly from the kidney of dead fish infected with *P. damselae* subsp. *piscicida* (Matsuoka *et al.*, 1986). It was modified slightly by using an acidometric method (Yasunaga *et al.*, 1985).

Toranzo *et al.* (1991) reported that the strain of *P. damselae* subsp. *piscicida* which was isolated from fingerlings of gilthead cultured in a marine farm in Spain showed sensitivity to ampicillin, chloramphenicol, tetracycline, oxolinic acid and trimethoprim-sulfamethoxazole. The strain was resistant to erythromycin, KM, SM and sulfonamide. Transferable R plasmid was not detected in strains isolated in Europe. Martinez-Manzanares *et al.* (2008) reported that TC- and OA-resistant strains of *P. damselae* subsp. *piscicida* were isolated in cultured Senegalese sole (*S. senegalensis*) and gilthead sea bream in Spain.

Transferable R plasmid has been detected in *P. damselae* subsp. *piscicida* isolated from hybrid striped bass in 1991 (Hawke *et al.*, 2003). The transferable R plasmid encoded with resistance to sulfonamide and tetracycline. Surprisingly, the drug-resistant genes of R plasmids in *P. damselae* subsp. *piscicida* were the same as those in human, domestic animal and fish pathogenic bacteria, except for the florfenicol-resistant gene. The *P. damselae* subsp. *piscicida* multiple drug-resistant strains not only increase drug selective pressure but also transfer R plasmid from the environment, domestic and human bacteria to fish-pathogenic bacteria in fish farms. These multiple drug-resistance clusters are constructed by transposon and are carried in different gene cassettes by integron. These transposons and integrons play an important role in the dissemination of multiple antimicrobial resistance genes in *P. damselae* subsp. *piscicida.*

Immunostimulants

Immunostimulators trigger the immune system by inducing or increasing its activity. The administration of hen egg lysozyme and ß-glucan as immunostimulator increased the resistance to pasteurellosis in yellowtail and gilthead sea bream, respectively (Hamaguchi and Kusuda, 1991; Noya *et al.*, 1995b). However, other immunostimulators such

as schizophyllan and scleroglucan (Matsuyama *et al.*, 1992) and M-glucan, chitin, Freund's complete adjuvant and *V. anguillarum* bacterin (Kawakami *et al.*, 1998a) did not increase resistance of yellowtail to pasteurellosis.

Vaccination

IN JAPAN. For the past 28 years, many researchers have devoted their work to developing vaccines against pasteurellosis. Vaccination by injection, oral, immersion and the spray method using formalin-killed cells have been used in cultured yellowtail in Japan. Vaccination with these methods has provided high protection against *P. damselae* subsp. *piscicida.* Agglutinating antibody titre in serum increased remarkably 3 weeks after vaccination. The titre of the skin mucus also increased 3 weeks after vaccination with the spray and immersion method (Fukuda and Kusuda, 1981a,b, 1985). In addition, 3×immersion vaccination and the combination of immersion and oral method were very effective against *P. damselae* subsp. *piscicida* (Kusuda and Hamaguchi, 1987). The effectivity of the vaccine depended on its cultivation period. Hamaguchi and Kusuda (1988) observed that the most effective vaccines were those that were cell cultivated for 48 h then killed by formalin. They also found that the attenuated live vaccine was most protective in pasteurellosis in yellowtail (Kusuda and Hamaguchi, 1988). Kusuda *et al.* (1988b), on the other hand, developed an effective ribosomal antigen vaccine from *P. damselae* subsp. *piscicida* against pasteurellosis in yellowtail.

A potassium thiocyanate extract (PTE) and an acetic acid-treated naked bacteria (NB) from a virulent strain of *P. damselae* subsp. *piscicida* have been used for vaccination. Yellowtail vaccinated with a mixture of PTE and NB showed higher resistance than those vaccinated with PTE or NB only (Muraoka *et al.*, 1991).

Kawakami *et al.* (1997) developed lipopolysaccharide-mixed, chloroform-killed cell vaccine against pasteurellosis in yellowtail. This vaccine provided protection against artificial challenge and natural infection by *P. damselae* subsp. *piscicida.* The phagocytic activity and index of blood and kidney leucocytes increased significantly in yellowtail injected with vaccine. The production of superoxide anion of kidney leucocytes also increased (Kawakami *et al.*, 1998b). Gravningen *et al.* (2008), on the other hand, developed an oil-based vaccine. They intraperitoneally injected cultured yellowtail, challenged and observed for 15 weeks, and found that the cumulative mortality of the vaccinated groups was lower than the non-vaccinated group. The vaccinated fish showed an increase of agglutinating antibody titre against *P. damselae* subsp. *piscicida*, as well as phagocytic activity and production of superoxide anions in leucocytes compared with non-vaccinated fish after vaccination. Many reports concerning the efficacy of vaccines against *P. damselae* subsp. *piscicida* have been published. However, commercial vaccines have not been produced in Japan since 1981. Recently, divalent vaccine with formalin-killed bacterin *P. damselae* subsp. *piscicida* and *Lactococcus garvieae* has been marketed in Japan.

IN THE USA. The attenuated strain (*AroA* mutant) has been constructed from virulent strains of *A. salmonicida* subspecies *salmonicida* (Vaughan *et al.*, 1993) and *E. ictaluri* (Thune *et al.*, 1999). These mutants have their pathway of aromatic amino-acid biosynthesis, known as the shikimate pathway, interrupted. *AroA* mutant was found to be effective as a live vaccine against the pathogens. Thune *et al.* (2003) also developed *aroA* mutant for live vaccine against *P. damselae* subsp. *piscicida* in hybrid striped bass. This attenuated vaccine provided significant protection against the disease.

IN EUROPE. Several types of vaccines against *P. damselae* subsp. *piscicida* have worked effectively on gilthead sea bream, sea bass, sole and red porgy (*Pagrus pagrus*) in European countries. The developmental stage of the fish is important for vaccination when using immersion toxoid-enriched, whole-cell vaccine of *P. damselae* subsp.

piscicida (WCEB bacterin) in gilthead sea bream. The immunization of small fish (0.5 g mean body weight) with WCEB increased the protection level effectively against artificial infection compared with bigger fish (5 g) (Magariños *et al.*, 1994a,b). The vaccination of 0.1 g and 1 g fish was also highly protected by immersion with a relative per cent survival (RPS) 70% higher than in all experiments (Moriñigo *et al.*, 2002). Dos Santos *et al.* (2001a) observed that specific antibody secreting cells (ASC) appeared in the spleen, head kidney and gut of juvenile sea bass after primary immunization (Photogen bacterin, Aqua Health Europe Ltd, Stirling, Scotland, UK) through intraperitoneal (i.p.) administration, and the number of specific ASC increased after secondary immunization. Significantly higher numbers of ASC were observed in the gill of sea bass fry (with an initial weight of 2 and 5 g) immunized by direct immersion in a *P. damselae* subsp. *piscicida* bacterin (Photogen), as compared with smaller fry (with an initial weight of 0.5 g) (Dos Santos *et al.*, 2001b). Specific serum antibody was also observed when live and heat-killed cells of *P. damselae* subsp. *piscicida* were injected on to the sea bass (Bakopoulos *et al.*, 1997a). Mulero *et al.* (2008) demonstrated that the immunocompetence of the gilthead sea bream during early developmental stages depended on innate immunity, as well as those of zebrafish (*Danio rerio*) and other teleosts (Zapata *et al.*, 2006). It was suggested that vaccination of young larvae might be unsuccessful because the lymphocyte marker genes (including Rag1/2, T-cells receptor alpha/beta and immunoglobulin light and heavy chains) were not expressed in the early stages of young larvae after *P. damselae* subsp. *piscicida* bacterin immunization (Mulero *et al.*, 2008). When the sea bass broodstock were immunized with *P. damselae* subsp. *piscicida* and lipopolysaccharides (LPS) derived from *Escherichia coli* for 1 or 2 months before spawning, the RPS values of the larvae from immunized parents were very high; however, the RPS of vaccinated larva was low (Hanif *et al.*, 2005). It could be that maternal immunity prevented infection with *P. damselae* subsp. *piscicida*.

There are several published data on the effectiveness of divalent vaccines of *P. damselae* subsp. *piscicida* and *Vibrio* species. Gravningen *et al.* (1998) showed the effectiveness of a divalent vaccine for sea bass by immersion and booster against infection with *V. anguillarum* and *P. damselae* subsp. *piscicida* at 7 weeks post-revaccination. Moriñigo *et al.* (2002) showed that immunization with divalent vaccine formalin-killed whole cells of *P. damselae* subsp. *piscicida* and *V. alginolyticus* in the gilthead sea bream (0.1 g and 1 g mean body weight) was highly effective, with over 70% RPS in all experiments. Combinational vaccination of immersion and booster with a divalent vaccine of formalin-killed *P. damselae* subsp. *piscicida* and *V. harveyi* bacterins also provided a high protection in the sole with a similar level of the i.p. injection to their monovalent bacterins (Arijo *et al.*, 2005). Afonso *et al.* (2005) observed the peritoneal response of sea bass from day 1 to 11 months post-injection with monovalent and divalent (*P. damselae* subsp. *piscicida* and/or *V. anguillarum*) vaccine formulations with or without oil adjuvant. A steady increase occurred in lymphocytes/small cells and eosinophilic granular cells, while a high concentration of neutrophils and macrophages was maintained from 30 to 60 days in groups injected intraperitoneally with adjuvant formulations with the vaccine. The additional treatment of Freund's incomplete adjuvant (FIA) also demonstrated effective results in gilthead sea bream (Acosta *et al.*, 2005). The cumulative mortality of juvenile gilthead sea bream vaccinated with formalin-killed *P. damselae* subsp. *piscicida* bacterin in FIA was lower than that of using bacterin alone. The nitric oxide (NO) response of kidney macrophages in the juvenile gilthead sea bream vaccinated with *P. damselae* subsp. *piscicida* bacterin alone was significantly higher than that of non-vaccinated fish, while that of bacterin plus FIA resulted in a further significant enhancement. Acosta *et al.* (2005) suggested that the upregulation of the NO response by

the vaccine might be an implication of increased protection.

Antigenic proteins on the *P. damselae* subsp. *piscicida* surface are very important to induce fish immune responses. The outer membrane was the most immunodominant antigen and produced an immunological response to gilthead sea bream (Arijo *et al.*, 2004). It may be useful to design prophylactic strategies that include a vaccine. Nitzan *et al.* (2004) demonstrated that a *P. damselae* subsp. *piscicida* vaccine prepared under the growth condition of 2.5% NaCl medium at 25°C was highly effective against *P. damselae* subsp. *piscicida* infection in the hybrid bass because its envelope was produced abundantly in the culture medium containing 2.5% salinity (Nitzan *et al.*, 2004). In the sea bream, red porgy and sea bass, the immune serum killing by the classical complement pathway may provide some degree of protection against Photobacteriosis, but enhanced expression of the capsule by *P. damselae* subsp. *piscicida in vivo* may restrict complement-mediated killing, especially in immunized sea bass (Acosta *et al.*, 2006).

Pseudomonas Species

Taxonomy of pseudomonads

Pseudomonads exist throughout the aquatic environment and are associated with both healthy (Evelyn and McDermott, 1961; Bullock and Sniesko, 1969) and diseased fish. It is generally believed that these bacteria can be opportunistic pathogens or secondary infections. For example, when carp (*Cyprinus carpio*) are infected with *A. salmonicida* ssp. *nova*, the causative agent of carp erythrodermatitis, both pseudomonads and motile aeromonads are isolated readily from the internal viscera (Evenberg *et al.*, 1988). *Pseudomonas fluorescens* cause white nodules in the spleen and abscesses in the swim-bladder of tilapia (*Sarotherodon niloticus*) (Miyashita, 1984). Natural mortalities were highest when water temperatures were 15–20°C. Intramuscular injections of the bacteria caused mortalities and pathology similar to those observed in naturally infected fish. Granulomas were found in the spleen, liver and kidney (Miyashita *et al.*, 1984). The same bacterium has been reported to cause mortalities in 2-week-old tilapia fry (Duremdez and Lio-po, 1985) and causes disease in a number of other fish species, including goldfish (*Carassius auratus*) (Bullock, 1965), tench (*Tinea tinea*) (Ahne *et al.*, 1982) and rainbow trout (Li and Fleming, 1967; Li and Traxler, 1971). Bullock *et al.* (1965) reported on the biochemical characterization of a number of isolates from diseased fish in the USA. *P.* (*Alteromonas*) *putrefaciens* was identified as the causative agent of a disease outbreak in cultured rabbit fish (*Siganus rivulatus*) in the Red Sea (Saeed *et al.*, 1987). Moribund fish were discoloured, had haemorrhagic necrosis on the body and mouth and exhibited exophthalmia and frayed fins. The authors were able to reinfect and kill fish when 10^9 bacteria were injected intraperitoneally into healthy rabbit fish (Saeed *et al.*, 1987, 1990). *P. chlororaphis* has been reported to cause high mortalities in Amago trout (*O. rhodurus*) in a hatchery in Japan (Hatai *et al.*, 1975), where fish exhibited large amounts of ascitic fluid and haemorrhaging in various parts of the body.

P. pseudoalcaligenes was isolated from skin lesions on rainbow trout infected with *Y. ruckeri* type I (Austin and Stobie, 1992b). Experimental injection of 10^5 cells of *P. pseudoalcaligenes*, either intraperitoneally (i.p.) or intramuscularly (i.m.), resulted in total mortalities within 7 days. Intramuscular injection resulted in some muscle liquefaction around the injection site. *P. putida* and *P. luteola* have also been implicated in disease of rainbow trout in Turkey and Japan (Altinok *et al.*, 2006, 2007).

Pseudomonas plecoglossicida

P. plecoglossicida causes disease in ayu (*Plecoglossus altivelis*) and pejerrey (*Odonthestes bonariensis*). Bacterial haemorrhagic ascites (BHA) of ayu often occurs a short

time after naturally or artificially produced fish are introduced into culture ponds and concurrently at any developmental stage during culture, particularly after chemotherapy for *F. psychrophilum* infection (Park *et al.*, 2000). Gross pathological signs for dead and moribund fish include pale body discoloration, distended abdomen due to ascites, pale gill coloration, bloody ascites fluid in the peritoneal cavity, splenomegaly, swollen kidney and occasional haemorrhage in the abdominal adipose tissue. Histologically, the most prominent pathological changes are noted in the spleen and kidney (Kobayashi *et al.*, 2004). *P. plecoglossicida* causes serious damage to the ayu culture industry in Japan. The cumulative mortality may reach 30–50% (Kobayashi *et al.*, 2004). As for all diseases, reducing predisposing factors, such as overcrowding and overfeeding, is important (Park *et al.*, 2000).

P. plecoglossicida are motile, Gram-negative rods and growth occurs between 10 and 30°C in the presence of 0–5% NaCl. Catalase and cytochrome oxidase are produced. Arginine dihydrolase is produced, whereas lysine and ornithine are not decarboxylated. Hydrolysis of gelatin, starch and Tween 80 is negative. The bacteria are haemolytic on blood agar. The G+C content of the DNA is 62.8 mol % (Nishimori *et al.*, 2000). The bacterium grows on basic laboratory media such as TSA and blood agar, where it is haemolytic. Isolation is done at 25°C. The bacterium can be identified readily by slide agglutination with specific rabbit serum (Nishimori *et al.*, 2000; Park *et al.*, 2000; Kobayashi *et al.*, 2004). Recently, Izumi *et al.* (2007) described a PCR amplification technique specific for *P. plecoglossicida* that targeted the DNA gyrase, gyrB. The authors were able to detect specifically that the pathogen was in both the kidney and intestine of ayu.

At present, there are no licensed chemotherapeutic compounds that are effective against BHA of ayu (Kobayashi *et al.*, 2004). Park *et al.* (2000) and Park and Nakai (2003) showed that oral administration of phage-impregnated feed to ayu resulted in protection against experimental infection with *P. plecoglossicida*.

Pseudomonas anguilliseptica

Species of fish affected and geographical distribution

Several pseudomonads have been isolated from fish but the most significant is *P. anguilliseptica*. This bacterium was first described as the aetiological agent of 'Sekiten-byo', or red-spot disease of Japanese eels (*Anguilla japonica*), by Wakabayashi and Egusa (1972). Since that time, it has been isolated from European eel (*A. anguilla*) (Stewart *et al.*, 1983), black sea bream (*Acanthopagrus schlegeli*) (Nakajima *et al.*, 1983), ayu (Nakai *et al.*, 1985c), Atlantic salmon (*Salmo salar*), sea trout (*S. trutta*), rainbow trout, whitefish (*Coregonus* sp.) and Baltic herring (*Clupea harengus membras*) (Wiklund and Bylund, 1990; Lonnstrom *et al.*, 1994; Wiklund and Lonnstrom, 1994), cod (*Gadus morhua*) (Ferguson *et al.*, 2004), gilthead sea bream (*S. aurata*), sea bass (*D. labrax*), turbot (Berthe *et al.*, 1995) and possibly the giant sea perch (*Lates calcarifer*) and the estuarine grouper (*Epinephelus tauvina*) (Nash *et al.*, 1987). Experimental challenges have caused mortalities in bluegill sunfish (*Lepomis macrochirus*), goldfish, carp and loach (*Misgurnus anguillicaudatus*) (Muroga *et al.*, 1975). The disease has been found in Japan (Wakabayashi and Egusa, 1972), Scotland (Stewart *et al.*, 1983), Taiwan (Nakai *et al.*, 1985b), and most recently in Finland (Wiklund and Bylund, 1990) and France (Berthe *et al.*, 1995).

The disease

The disease was first known as 'Sekiten-byo', or red-spot disease of Japanese eel, where it could cause significant loss of cultured fish. Clinical signs of the disease appear to be the same in all species affected, namely petechial haemorrhage of the skin, peritoneum and liver. The kidney can be affected severely, with some liquefactive necrosis (Wakabashi and Egusa, 1972; Ellis *et al.*, 1983; Wiklund and Bylund, 1990).

The microorganism

P. anguilliseptica is a Gram-negative, motile rod, cytochrome oxidase-positive, producing

no acid from glucose (negative in Hugh Liefson's O/F medium), is negative for indole, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, but utilizes citrate and usually produces gelatinase. The Finnish isolates, unlike others described to date, do not hydrolyse Tween 80. The bacterium grows slowly on tryptone soya agar (TSA), forming round, greyish convex colonies of 1 mm diameter after 3–4 days of incubation at 25°C. It will grow at temperatures ranging from 4 to 30°C (with no growth at 37°C) and at salinities of 0–4%. Stewart *et al.* (1983) have estimated the mol % guanine plus cytosine (G+C) content of the type strain NCMB 1949 to be 57.4% and their Scottish isolate to be 56.5 mol % G+C. Accepted ranges are 55–64% for *Pseudomonas* versus 38–40% for pseudomonads reclassified as *Alteromonas*. The whole-cell fatty acid analysis of 79 strains of *P. anguilliseptica* demonstrated that all were related but distinct from other pseudomonads (Nakajima *et al.*, 1983). Three different serotypes of *P. anguilliseptica* have been identified, based on the presence or absence of a heat-labile K antigen. There are a K^+ and a K^- serotype isolated from Japanese eels (Nakai *et al.*, 1981) and a separate K^+ serotype from ayu (Nakai *et al.*, 1985b). The Finnish isolates are similar to the serotype isolated from ayu (Wiklund and Bylund, 1990). Outer membrane protein patterns appear to be useful for showing the similarity among strains (Nakajima *et al.*, 1983). Lopez-Romalde *et al.* (2003b) and Romalde *et al.* (2003) have described two O-serotypes. Serotype O1 is associated with fish isolates from turbot, sea bream, sea bass, herring and salmonids, whereas serotype O2 is associated with Japanese and European eel isolates.

Diagnostic methods

The bacterium will grow on basic laboratory media, such as brain–heart infusion (BHI) agar, TSA and blood agar, but will not grow on *Pseudomonas* isolation agar (Difco laboratories) (Wiklund and Bylund, 1990). The bacterium can be identified readily using slide agglutination with specific rabbit serum, but it is important to dilute the serum 1:10 to prevent cross-reactions with other pseudomonads or *V. anguillarum* (Nakai *et al.*, 1981; Toranzo *et al.*, 1987). Lopez-Romalde *et al.* (2003a) have recommended the use of the API20NE miniaturized bacterial identification system. Consistent results were achieved only when the bacteria were grown on blood agar and adjusted spectrophotometrically to an optical density of 1 (at an absorbance of 580 nm).

Control and treatment

The disease can be eliminated effectively in eels by raising the water temperature to 26–27°C (Muroga *et al.*, 1975; Stewart et *al.*, 1983). This method of control cannot be applied to rainbow trout or other fish species that do not tolerate high temperatures. It is postulated that this treatment is effective because the bacterium cannot grow at these temperatures and the bacteria are lysed more quickly by normal eel serum at 28°C than at 20°C (Nakai *et al.*, 1985a). Wiklund and Bylund (1990) attempted to treat Atlantic salmon with oxytetracycline (75 mg/kg for 8 days), but this had only a limited effect. They found that ampicillin and trimethoprim/sulfamethoxazole were the most effective under *in vitro* conditions.

Pathogenesis and immunity

Nakai and Muroga (1979) studied the immune response of Japanese eels to formalin-killed *P. anguilliseptica* bacterin, with or without Freund's complete adjuvant. The fish were injected twice i.m., at 1-week intervals, at doses of 1 mg/100 g body weight. Higher serum agglutinating titres were obtained in the fish receiving the bacterin with adjuvant. Fish vaccinated, held in fresh water at 19–25°C and then placed in outdoor tanks for the winter were refractory to an artificial challenge 5 months later. Since this disease is most prevalent in the spring, vaccination seems to be a good method for preventing the disease.

Francisella Species

Recently, the Gram-negative facultative, intracellular, fastidious bacterium *Francisella* has been implicated as a new fish pathogen in both freshwater and marine fish.

Species of fish affected and geographical distribution

A number of fish species have been shown to be infected and diseased with *Francisella* sp. These include the three-line grunt (*Parapristipoma trilineatum*) from Japan (Kamaishi *et al.*, 2005), tilapia (*Oreochromis* spp.) from America and Asia (Hsieh *et al.*, 2006; Kay *et al.*, 2006; Maule *et al.*, 2007), Chilean Atlantic salmon (Birkbeck *et al.*, 2007), Norwegian cod (*G. morhua*) (Nylund *et al.*, 2006; Olsen *et al.*, 2006b), a number of ornamental cichlids (Hsieh *et al.*, 2007) and American hybrid striped bass (*M. chrysops* × *M. saxatilis*) (Ostland *et al.*, 2006).

The disease

Infected fish exhibit clinical signs such as erratic swimming, lost of appetite, exophthalmia and dark pigmentation. The disease is characterized by numerous white, granulomatous cysts in the visceral organs (kidney, spleen, liver and heart), gills and skin (Hsieh *et al.*, 2006; Nylund *et al.*, 2006; Ottem *et al.*, 2008; Soto *et al.*, 2009).

The microorganism

Francisella are Gram-negative, non-motile coccobacilli (Mikalsen *et al.*, 2007; Ottem *et al.*, 2007a,b, 2009). The two species of *Francisella* that caused disease in fish were *F. piscicida* (Ottem *et al.*, 2007a,b) and *F. philomiragia* subsp. *noatunensis* (Mikalsen *et al.*, 2007); however, Ottem *et al.* (2009) and Mikalsen and Colquhoun (2009) showed that these two species were indistinguishable and renamed them *F. noatunensis*. Ottem *et al.* (2009) also demonstrated that a Japanese isolate of *Francisella* sp. (Kamaishi *et al.*, 2005) from the three-line grunt was a distinct subspecies that they classified as *F. noatunensis* subsp. *orientalis*. Mikalsen and Colquhoun (2009), however, propose that this isolate is a distinct species, *F. asiatia*. Another species, *F. victoria*, has been discussed as a pathogen of tilapia by Kay *et al.* (2006), but there is little information about this isolate except for characterization of the lipopolysacharide. Its relationship to the other fish isolates is unclear. *Francisella* spp. isolated from fish grow best on cysteine heart agar with 5% sheep blood. Their optimum growth temperature is 20–25°C with a range of 10–25°C. Further characteristics can be found in Mikalsen *et al.* (2007) and Ottem *et al.* (2007a,b, 2009).

Diagnostic methods

It has not been possible to culture all *Francisella* that have been implicated in disease. Many isolates have been identified using PCR, through the amplification and sequencing of the bacterial 16S rRNA genes from diseased tissue (Ostland *et al.*, 2006; Hsieh *et al.*, 2007; Maule *et al.*, 2007; Ottem *et al.*, 2008). When successful, the culturing of *Francisella* from fish lesions has been accomplished best using cysteine heart agar (CHA) plus 5% sheep red blood cells (Birkbeck *et al.*, 2007) or CHA plus rabbit blood (Soto *et al.*, 2009), Thayler Martin agar (Hsieh *et al.*, 2006) or modified Thayler Martin agar (Soto *et al.*, 2009), blood agar plates with 0.1% cysteine and 1% glucose (Nylund *et al.*, 2006) or, in some cases, using the CHSE-214 fish cell line (Hsieh *et al.*, 2006).

Control and treatment

There is currently limited information on the use of antibiotics and immunotherapy for this disease. *F. noatunensis* is resistant to trimethoprim-sulfamethazole, penicillin, ampicillin, cefuroxine and erythromycin,

and is susceptible to ceftazidimine, tetracycline, gentamicin and ciprofloxacin under *in vitro* conditions (Ottem *et al*., 2007a,b). Recently, Schroder *et al*. (2009) intraperitoneally vaccinated 30 g cod experimentally with *Francisella* sp. in an oil adjuvant. The fish had significant antibody responses to the *Francisella* sp., as shown using ELISA and immunoblotting. However, the role of antibodies in the cod immune system has not been determined (Schroder *et al*., 2009).

Enterobacterial Pathogens

The most important members of the family Enterobacteriaceae that are pathogenic to fish are the enteric redmouth disease agent, *Y. ruckeri* (see Chapter 13, this volume) and two species of *Edwardsiella*, *E. tarda* and *E. ictaluri* (Chapter 14). There are only a few reports suggesting that other enterics may be pathogenic for fish. However, recent reports suggest that they may represent an emerging problem. *Serratia*, *Proteus* and *Citrobacter* have all been implicated as potential fish pathogens. These bacteria share some common characteristics with other members of the Enterobacteriaceae: they are small, Gram-negative rods, catalase-positive and oxidase-negative and produce acid from glucose by oxidative and fermentative metabolism. Motile species have peritrichous flagella and most reduce nitrate to nitrite. Further biochemical characterization can be carried out using the various commercial test strips that are available.

Proteus (*Providencia*)

Proteus rettgeri (reclassified as *Providencia rettgeri* in the 1984 *Bergey's Manual*; Penner, 1984) was isolated consistently from the internal organs and lesions of silver carp (*Hypophthalmichthys molitrix*) in Israel (Bejerano *et al*., 1979). The disease appeared as an acute bacterial septicaemia, with severe mortalities occurring within 3 days when pond water temperatures were 20°C or higher, compared with 8 days at lower temperatures. Some ulceration and muscle degeneration were observed and there were large, red and often deep ulcerative lesions on the external surface. Intramuscular injection of the isolates of *P. rettgeri* or exposure of scarified fish to bacteria produced mortalities and lesions, although with a lower level of mortality and less severe lesions than in natural infections.

Serratia

Serratia liquefaciens and *S. plymuthica* have been associated with bacterial septicaemia and mortalities in salmonid fish. *S. liquefaciens* was the predominant isolate recovered from dead and dying Atlantic salmon in Scottish marine cages (Mcintosh and Austin, 1990). The internal organs were affected, particularly the kidney, spleen and liver, but there were no external signs of infection. Starliper (2001) described *S. liquefaciens* as a pathogen of laboratory reared Arctic charr (*Salvelinus alpinus*). The only external sign of disease was redness and swelling of the anus. Internally, tissues were severely haemorrhagic. Aydin *et al*. (2001) reported that diseased rainbow trout lost scales, had bloody and swollen kidney and had haemorrhagic spots on the gills and bloody exudates in the intestine. The bacterium was also a common isolate from the internal organs of lake trout (*S. namaycush*) and brook trout (*S. fontinalis*) from a Canadian hatchery with a low but inconsistent level of mortalities (S. Lord, 1991, unpublished observations). An axenic culture of the bacterium has also been isolated from an ulcerative skin lesion on a wild rock bass (*Ambloplites rupestris*) (J. Daly, 1985, unpublished observations). In all these cases, the results of biochemical tests on the isolates closely resembled those reported for *S. liquefaciens* (Grimont and Grimont, 1984). Initial diagnosis of the Atlantic salmon isolate was difficult, due to its anomalous oxidase reaction, and arrangement of flagella suggested that it was an aeromonad (Mcintosh and Austin, 1990). When

injected into Atlantic salmon, the isolate caused pronounced damage to the musculature at the site of injection and rapid mortalities (72 h for an infective dose of 10^3 bacteria). Starliper (2001) reported that the LD_{50} ranged from 1.8×10^8 to 1.41×10^6 cfu/fish for Atlantic salmon and Arctic charr. Rainbow trout also showed muscle and internal organ necrosis when injected with 10^7 bacteria, but mortalities did not occur (Mcintosh and Austin, 1990). Extensive, spreading muscle necrosis can appear when *S. liquefaciens* is injected subcutaneously into rainbow trout (S. Lord, 1991, unpublished observation), perhaps due to the action of the many exoenzymes produced by this bacterium. Vigneulle and Baudin Laurencin (1995) described *S. liquefaciens* as a pathogen of turbot that caused low but continuous mortality in marine cages in France. Externally, there were no clinical signs, except for a hyperpigmentation of the skin. Internally, there was abundant ascites fluid and both the kidney and spleen were swollen.

S. plymuthica, a red pigmented species, was isolated repeatedly from moribund rainbow trout fingerlings in a Spanish hatchery (Nieto *et al.*, 1990). There were no external signs of disease and the organism was detected after bacteriological culture of the organs. A mean lethal dose (LD_{50}) of 10^5 cells was reported for i.p. injections into rainbow trout and again there were signs of tissue damage from the challenge. Austin and Stobie (1992b) isolated *S. plymuthica* from skin lesions on rainbow trout which were otherwise infected with *Y. ruckeri* type I. Experimental injection of 10^4 cells of *S. plymuthica*/fish, either i.m. or i.p., resulted in total mortality within 7 days. The bacterium caused severe erosion of the skin and musculature. Baya *et al.* (1992) showed that *S. marcescens* could also potentially be a fish pathogen. These authors isolated the bacterium from the kidney of healthy white perch. The bacterium has an LD_{50} of 5×10^3 for rainbow trout and 1×10^5 for striped bass, regardless of whether the bacteria are given i.p. or i.m. Remarkably, the fish died sooner when the i.m. route was used. The LD_{50} of the ECP was 0.8 µg protein/g fish for striped bass and 0.4 µg protein/g fish for rainbow trout. Starliper (2001) found that *S. liquefaciens* was susceptible to nalidixic acid, neomycin, oxolinic acid, Romet, streptomycin and trimethoprim. His isolates were resistant to ampicillin, colistin, nitrofurantoin, sulfadiazine and tetracycline. Aydin *et al.* (2001) found that chloramine-T and potentiated sulfonamide were effective treatments during a disease outbreak in rainbow trout. Similarly, the turbot isolate of Vigneulle and Baudin Laurencin (1995) was susceptible to chloramphenicol, trimethoprim-sulfamethoxazole, flumequin, furans, oxytetracycline and oxolinic acid.

Other enteric bacteria

Based on the above reports, some species of *Serratia* and *Proteus* appear to be opportunistic pathogens of fish. Not only have they been found as the predominant isolate from diseased fish, but also they produce disease in fish exposed to them experimentally. Other species of enteric bacteria, notably *Citrobacter freundii* (Sato *et al.*, 1982; Baya *et al.*, 1990b; Sanz, 1991; Karunasagar *et al.*, 1992) and *Enterobacter agglomerans* (Hansen *et al.*, 1990), have been suggested as possible fish pathogens, on the basis of isolations from infections and mortalities. However, further evidence is needed to substantiate these species as more than incidental pathogens. Toranzo *et al.* (1994) found that nine fish isolates were of low virulence (LD_{50} of greater than 5×10^7) but could establish a carrier state within the fish. As enterobacteria are ubiquitous in the environment, it is not surprising that some should be opportunistic pathogens of fish. They would present a particular risk in warmwater ponds that frequently are exposed to faecal material, such as silver carp ponds fertilized regularly with poultry manure (Bejerano *et al.*, 1979) or other polluted waters. A case in point is the recent report of a *C. freundii* infection of carp fingerlings. This may have resulted from a contaminated water supply after the onset of the monsoon (Karunasagar

et al., 1992). Baya *et al.* (1992) have reported that fish living in river water that receives city sewage carry a significantly higher number of bacterial species in comparison with fish from less polluted waters.

Atypical Lactic Acid Bacteria

This section discusses atypical lactic acid bacteria that have been implicated in pathological conditions of fish. Lactic acid bacteria have been shown to mediate disease in fish that have undergone some kind of stress and are usually associated with post-spawning mortalities. Ross and Toth (1974) first reported the condition of pseudokidney disease associated with a *Lactobacillus* sp. Hiu *et al.* (1984) classified some isolates into a new species, *L. piscicola*, and later Collins *et al.* (1987) reclassified *L. piscicola* into a new genus, *Carnobacterium piscicola*. Mora *et al.* (2003) have further reclassified the *C. piscicola* species as *C. maltaromaticum*. Wallbanks *et al.* (1990) have classified some fish lactic acid bacteria into the new genus and species *Vagococcus salmoninarum*.

Species of fish affected and geographical distribution

Bacteria belonging to the genus *Lactobacillus* or *Carnobacterium* have been isolated from the following species of fishes with disease conditions: rainbow trout (Cone, 1982), lake trout (*S. namaycush*) (Daly and Stevenson, 1986), cutthroat trout (*S. clarki*) and Chinook salmon (*O. tshawytscha*) (Hiu *et al.*, 1984), brown trout (*S. trutta*) and carp (Michel *et al.*, 1986), striped bass (*M. saxatilis*) and channel catfish (Baya *et al.*, 1991). The bacterium has also been isolated from brown bullheads (*I. nebulosus*) that showed no signs of disease (Baya *et al.*, 1991). It is widely distributed, having been isolated in Canada, the USA, Australia, Belgium and France (Cone, 1982; Hiu *et al.*, 1984; Michel *et al.*, 1986; Humphrey *et al.*, 1987).

The disease

A septicaemic condition of fish known as pseudokidney disease was first reported by Ross and Toth (1974) as having been caused by *Lactobacillus* sp. These authors and Cone (1982) reported that the disease was in female fish only. Cone (1982) reported the disease in 'overripe' fish that had not lost all their eggs after spawning. The bacterium was isolated from kidney and ascites. Gram staining revealed large numbers of Gram-positive bacteria both in ascites and within eggs. Herman *et al.* (1985) reported that the disease was in both sexes of fish. The fish had haemorrhaging at the base of the fins, and some males had hyperaemic skin lesions. Internally, many fish had ascites, pale liver and haemorrhage of the intestine and gonads, and some organs had grey membranes.

The microorganisms

Lactic acid bacteria have been reported from fish since first being described by Evelyn and McDermott (1961). Hiu *et al.* (1984) classified some isolates into a new species, *L. piscicola*, and later Collins *et al.* (1987) reclassified *L. piscicola* and *L. carnis* (which is isolated from meat) into a new genus, *Carnobacterium*, as C. *piscicola*. Mora *et al.* (2003) further reclassified the *C. piscicola* species as *C. maltaromaticum*. The bacteria are Gram-positive, non-spore-forming rods, occurring singly or in chains. They do not produce catalase or cytochrome oxidase, do not reduce nitrate and do not produce indole, urease, gelatinase, lysine decarboxylase or ornithine decarboxylase, but they possess arginine dihydrolase. All strains characteristically do not grow on acetate medium. Strains also differ in carbohydrate fermentation. The reader is referred to the articles by Hiu *et al.* (1984), Michel *et al.* (1986), Baya *et al.* (1991), Starliper *et al.* (1992) and Toranzo *et al.* (1993) for further description of biochemical profiles.

Members of the genus *Carnobacterium* differ from the true lactobacilli only in their

ability to grow on acetate media and their synthesis of oleic acid rather than ds-vaccenic acid (Collins *et al.*, 1987). Wallbanks *et al.* (1990) have classified some fish lactic acid bacteria into the new genus and species, *Vagococcus salmoninarum*. Schmidtke and Carson (1994) examined 13 isolates of *V. salmoninarum* from Australia and Norway and compared them with the isolates described by Wallbanks *et al.* (1990). They amended and extended the description of the bacterium and suggested that *V. salmoninarum* could be separated phenotypically from *L. piscium* and *C. maltaromaticum*, using tests for hydrogen sulfide (H_2S) production and a-haemolysis of sheep red blood cells and by examining for the production of acid from a battery of carbohydrates. Toranzo *et al.* (1993) suggested a series of tests to help differentiate *Lactobacillus* sp., *C. maltaromaticum*, *C. divergens*, *V. salmoninarum* and *L. piscium*. These include growth on acetate agar, production of arginine dihydrolase, acid production from mannitol and inulin, H_2S production and starch hydrolysis. Other lactic acid bacteria, such as those described by Starliper *et al.* (1992) and Austin and van Poucke (1993), are different from the known described species of fish-pathogenic lactic acid bacteria and have yet to be speciated.

Diagnostic methods

These bacteria can be isolated readily on TSA or BHI agar (Hiu *et al.*, 1984; Herman *et al.*, 1985; Baya *et al.*, 1991). Fermentation patterns can be determined quickly with the API 50 CHI test strips. Michel *et al.* (1986) and Baya *et al.* (1991) found that weak reactions with the API 50 CHI were difficult to read. *C. maltaromaticum* can be separated from the related *C. divergens* by the fermentation patterns for inulin and mannitol. *C. maltaromaticum* ferments both of these, while *C. divergens* does not (Montel *et al.*, 1991). Baya *et al.* (1991) showed that a limited number of *Carnobacterium* strains isolated from fish were serologically heterogeneous. These workers also showed that there were differences in outer membrane protein profiles of their isolates.

Williams and Collins (1992) designed synthetic oligonucleotide probes specific for the genus *Vagococcus*. Ruiz-Zarzuela *et al.* (2005) used a PCR test to detect *V. salmoninarum* in rainbow trout. Brooks *et al.* (1992) reported on the use of the PCR and oligonucleotide probes, designed from 16S rRNA sequence data, for the detection and identification of *Carnobacterium* from meat. One of their probes is specific for *C. maltaromaticum/C. gallinarum*. Specific probes, based on their 16S rRNA sequences, could not be designed to differentiate these two species, because of the 99% 16S rRNA sequence relatedness of the two species (Brooks *et al.*, 1992). To overcome this, alternative methods need to be developed. A note of caution is warranted when diseased animals are examined histologically using the Brown and Brenn Gram stain. One should be aware that the very important pathogen, *R. salmoninarum*, is also Gram-positive. This latter bacterium, however, is smaller in length.

Control and treatment

There is no information available on transmission of these atypical lactobacilli. Baya *et al.* (1991) found that their *Carnobacterium* isolates were resistant to antibiotics in common use in aquaculture, such as oxytetracycline, quinolones, nitrofurans and potentiated sulfonamides. Michel *et al.* (1986), Baya *et al.* (1991) and Toranzo *et al.* (1993) reported that their isolates were sensitive to erythromycin *in vitro*.

Pathogenesis and immunity

Ross and Toth (1974), Cone (1982), Herman *et al.* (1985), Michel *et al.* (1986) and Baya *et al.* (1991) all reported difficulty in transmitting the disease by i.p. injection of the bacterium. Either another challenge route is necessary or the bacterium causes

disease only when the animals are stressed or at a certain age. Baya *et al*. (1991) reported that the LD_{50} for the reference strain ATCC 35586, given i.p. to rainbow trout, was 2.0×10^6 cells, whereas striped bass were resistant to the bacterium at doses of 8.0×10^7. Every strain tested created a carrier state in the kidney of fish that survived the i.p. challenge. This was true for both striped bass and rainbow trout. Toranzo *et al*. (1993) reported a strain from rainbow trout that was unusually virulent. This isolate had an $LD_{50} < 5\times10^6$ after either an injection or a waterborne challenge.

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18 *Saprolegnia* and Other Oomycetes

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Introduction

Oomycete infections including *Saprolegnia* and *Aphanomyces* species are responsible for infections on fish in aquaculture, and descriptions date from the mid-18th century (Humphrey, 1893). These pathogens are commonly known as water moulds and as such are multicellular and assimilate nutrients by extracellular digestion. Oomycete infections are endemic to all freshwater fish around the world and are partly responsible for the decline of natural populations of salmonids and other freshwater fish. The Saprolegniaceae, in particular the genus *Saprolegnia*, are responsible for significant infections, involving both living and dead fish and eggs, particularly in aquaculture facilities. This is an example of disease that contributes to poor welfare and requires control. Oomycetes are classical saprophytic opportunists, multiplying on fish that are physically injured, stressed or infected (Pickering and Willoughby, 1982a). They are generally considered agents of secondary infection arising from compromised conditions such as bacterial infections, immunosuppression, poor husbandry and infestation by parasites and social interaction. However, there are several reports of oomycetes as primary infectious agents of fish (Willoughby, 1978; Pickering and Christie, 1980; Stueland *et al*., 2005) and their eggs (Walser and Phelps, 1993; Bruno and Wood, 1999). These can readily become pathogens, resulting in epizootics among salmonids and other teleosts (Hatai and Hoshiai, 1992, 1993; Bly *et al*., 1994; Diéguez-Uribeondo *et al*., 1996). *Saprolegnia* may occur anywhere on the body of fish, but normally appear as a conspicuous, circular or crescent-shaped, white, cotton-like mycelium, particularly around the head and the caudal, adipose and anal fins (Neish and Hughes, 1980; Willoughby, 1989; Noga, 1993a; Hussein *et al*., 2001). The infection may spread over the body by radial extension until adjacent lesions merge (Fig. 18.1).

The oomycetes are an economically important group of mycotic agents that affect salmonids and other teleosts. Most fish pathogens belong to the order Saprolegniales. Although eight genera (*Achlya*, *Aphanomyces*, *Calyptratheca*, *Leptolegnia*, *Leptomitus*, *Pythiopsis*, *Saprolegnia* and *Thraustotheca*) have been reported in naturally or artificially induced infections, only *Achlya*, *Aphanomyces* and *Saprolegnia* are significant in aquaculture (Aller *et al*., 1987; Hatai and Hoshiai, 1993; Bly *et al*., 1994). Some species are isolated from fish consistently and generally these are assigned to a single major cluster, which form a coherent, separate taxon, *Saprolegnia parasitica* Coker (synonym *S. diclina* Humphrey type

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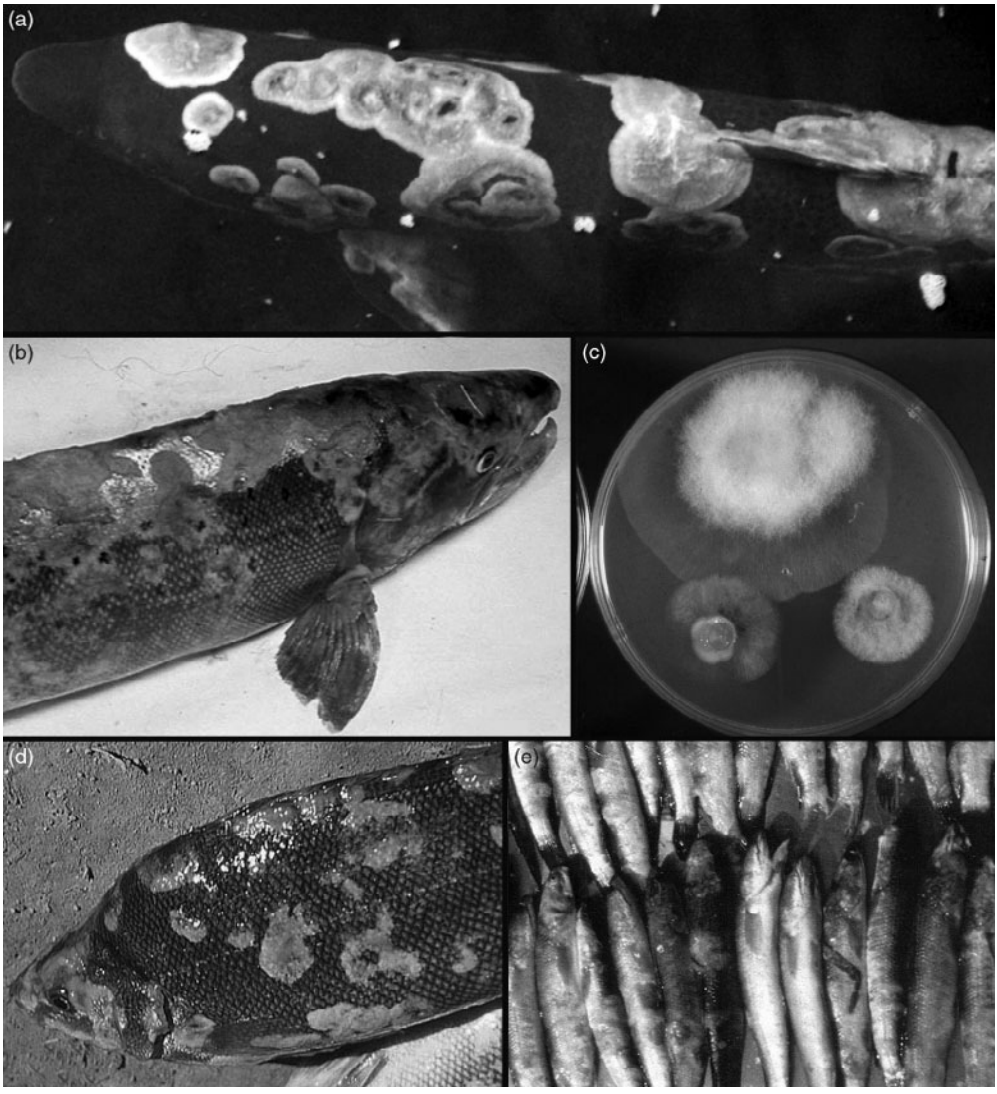


Fig. 18.1. Images of fish and eggs infected with oomycete pathogens. (a) A sluggish sea trout (*Salmo trutta*) photographed in the River Allen, Northumberland (UK), showing a heavy infection with *S. parasitica*. Note how the individual fungal colonies have merged and cover a significant proportion of the animal body. (b) An Atlantic salmon (*S. salar*) showing *S. parasitica* lesions on head and back. (c) Atlantic salmon eggs which have been challenged artificially with *Saprolegnia* spp. in the laboratory, showing typical cotton wool-like outgrowths. (d) A bony bream (*Nematalosa erebi*) infected with *S. parasitica* as a result of a 'winter saprolegniosis' outbreak in New South Wales, Australia [courtesy of Dr Matt Landos, NSW Vetinary Service]. (e) EUS affected snakeheads (*Channa* sp.) showing multiple dermal ulcers [courtesy of Dr James Lilley, Insitute of Aquaculture, Stirling, 1997].

1; Willoughby, 1978). The reader is referred to Taugboel *et al.* (1993), Diéguez-Uribeondo *et al.* (1994, 2007), Mueller (1994) and Bruno and Wood (1999) for reviews.

Several characteristics, including cell-wall composition, mitochondrial morphology and numerous sequences of conserved cytoplasmic genes, differentiate the biflagellated

water moulds from the true fungi. Attention has long been drawn to the similarity between the oomycetes and the chromophyte algae which contain chlorophyll a and c (Beakes, 1989; Barr, 1992) as these organisms share many common structural (flagellar rootlet organization and transitional helix conformation) and biochemical features (e.g. cellulosic walls, beta 1–3 glucan storage reserves). Molecular techniques are also improving our understanding of the overall taxonomic relationships within the oomycetes (see Beakes and Sekimoto, 2009). It is now apparent that the oomycetes evolved from holocarpic organisms that were parasites of seaweeds, crustaceans and nematodes (Sekimoto *et al.*, 2007, 2008a,b). It is apparent that the majority of early diverging oomycete genera are marine and this suggests this lineage evolved in the sea, contrary to Dick's (2001) conclusion that all evidence suggests the oomycetes are of freshwater or terrestrial origin.

Saprolegnian oomycetes have a complex life cycle (Fig. 18.2), involving sexual and asexual stages (Kanouse, 1932; Noga, 1993a). Sexual reproduction involves the production of gametangia, the male antheridium and the female oogonium (Fig. 18.3). Haploid antheridial male nuclei and egg nuclei arise following meiosis and fuse during the maturation of the resting oospores. Asexual reproduction may occur by the production of chlamydospores (also known as gemmae), but generally zoospores are produced. In the Saprolegniales, motile primary zoospores, produced by the zoosporangium, encyst within minutes to produce

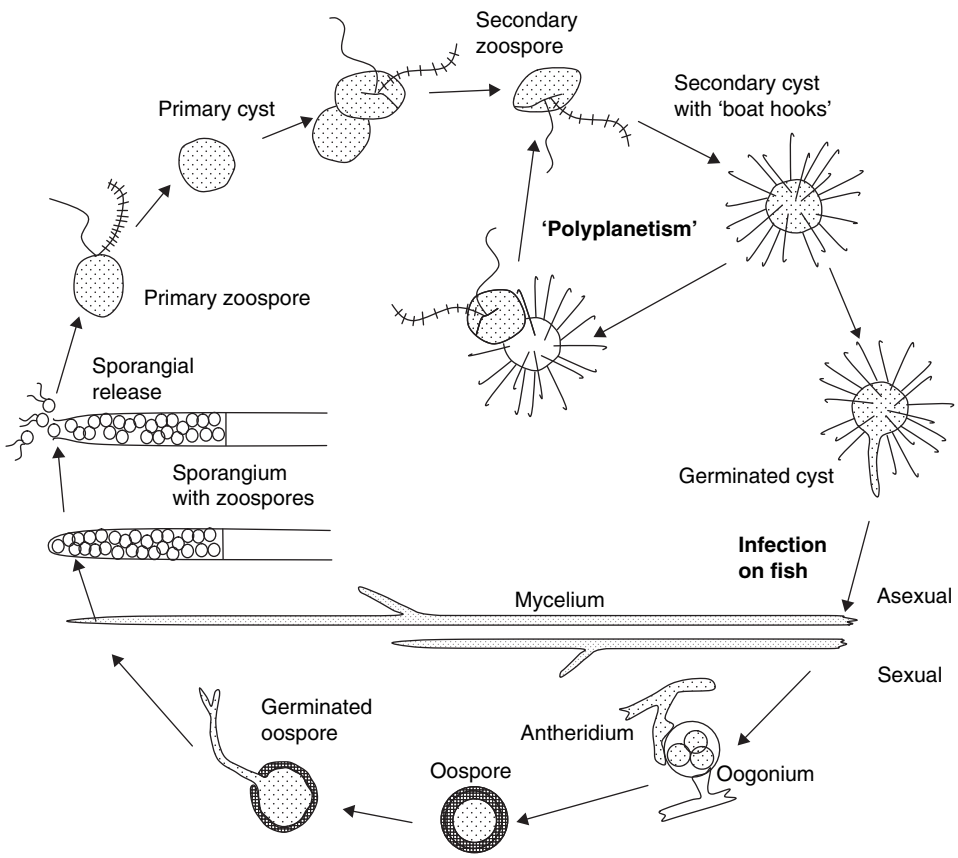


Fig. 18.2. Schematic diagram of the life cycle of *Saprolegnia parasitica* (adapted from van West, 2006).

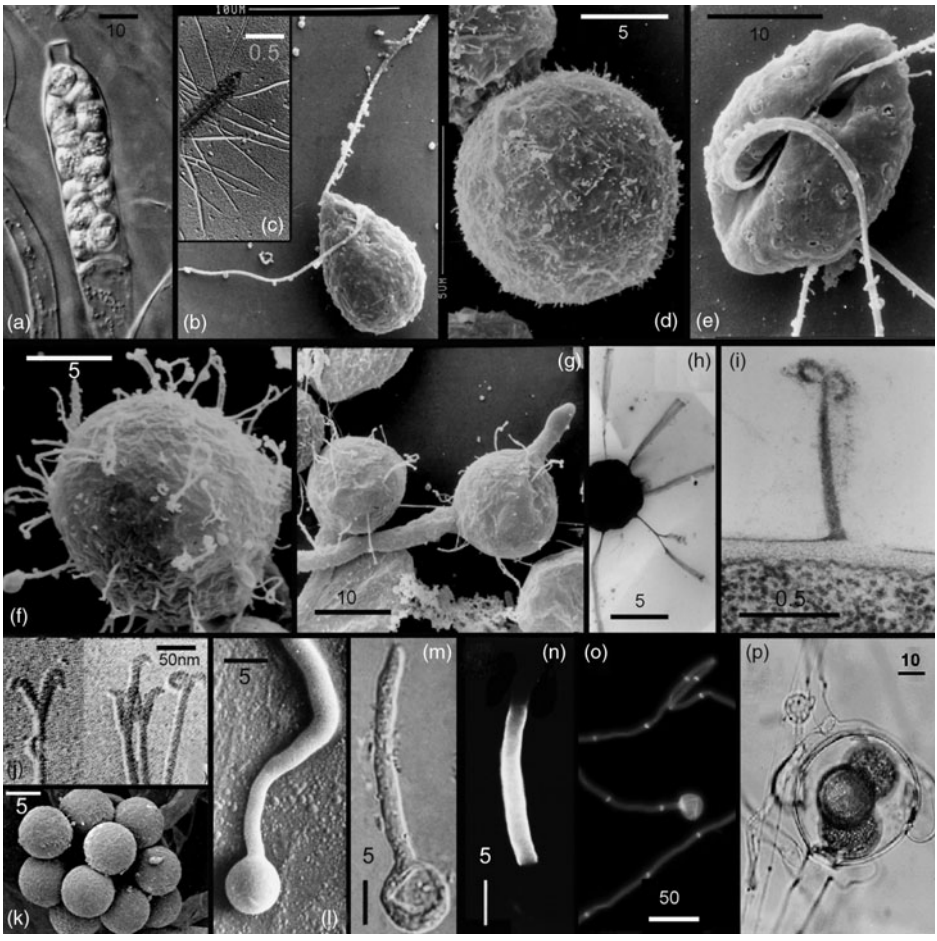


Fig. 18.3. General morphological characteristics of fish pathogenic oomycetes (a–p). Photographs of the stages in the asexual life cycle of *S. parasitica*. (a) Mature zoosporangium containing fully differentiated zoospores. (b) Pip-shaped primary zoospore. (c) Inset showing detail of anterior 'tinsel' flagellum, showing characteristic tripartite hairs which provide the forward thrust. (d) Primary cyst showing fine hair-like spines. (e) Reniform secondary zoospores showing ventral groove from which both anterior and posterior flagella arise. (f) Secondary cyst showing clusters of boathook spines, which cannot be clearly separated under SEM. (g) Germinating secondary cysts. (h) Shadowed wholemount secondary cyst showing characteristic bundles of long boathook spines which distinguish *S. parasitica* from other species. (i) Single short boathook spine decorating secondary cyst of the saprophytic species, *S. diclina*. (j) High power views of the spine tips from different isolates of *S. parasitica*, showing the variation in spine micromorphology. (k) The grape-like cluster of primary cysts at the mouth of the sporangial hypha of the EUS pathogen, *A. invadans*. (l) SEM of a germinating spore of *S. diclina* showing narrow initial germ tube, which is in close contact with the substrate. (m/n) Corresponding brightfield and fluorescence micrographs of germinating cysts of *S. parasitica*, staining with the lectin wheatgerm agglutinin (WGA), showing the sticky coat associated with the initial germ tube wall. (o) Fluorescence micrograph of a germling of *S. parasitica*, stained with the optical brightener calcofluor, showing the narrow septate germ tube, which is terminated by mini-sporangia (not shown). This pattern of germination is shown by isolates of the fish pathogen. (p) Typical diclinous oogonium showing oospores and antheridia derived from neighbouring hyphae. This oogonium is of the saprotroph *S. diclina*, but is within the morphological range shown by some isolates of *S. parasitica*. Figs a, c, k, o and p – D. Bruno unpublished photographs; figs b, d, e, f, g, l, m, n and o – Burr and Beakes (1994); fig. j – Beakes and Ford (1983); fig. h – Beakes (1983); fig. i – Beakes (1989).

primary cysts, which normally germinate indirectly to produce a secondary motile zoospore (Fig 18.3; Walker and van West, 2007). These more active secondary zoospores may swim for many hours before encysting to produce a secondary cyst. In turn, these may undergo further cycles of zoospore formation and encystment (so-called polyplanetism; Diéguez-Uribeondo *et al*., 1994) or may germinate to produce a hypha that may develop into a mycelium.

Oomycete outbreaks among farmed fish stocks frequently are associated with poor water quality, injuries associated with handling and grading, temperature shock and spawning. For many years, malachite green oxalate was used to control *Saprolegnia* outbreaks (Bailey, 1983a; Willoughby and Roberts, 1992a). Unfortunately, the potential teratogenic or mutagenic properties of malachite green (Meyer and Jorgenson, 1983; Clemmensen *et al*., 1984; Fernandes *et al*., 1991) have resulted in its use being banned in many countries (van West, 2006; Phillips *et al*., 2008). To date, only a limited number of chemical compounds show potential as oomyceticides and none are considered as effective as malachite green. Currently, the most successful strategy for the control of *Saprolegnia* in farmed fish is a combination of farm management, husbandry practices and chemical bath treatments including formalin. Generally, a combination of these practices is important in preventing or limiting oomycete outbreaks.

Histological changes resulting from oomycete infection include loss of integrity of the integument, oedema and degenerative changes in the muscle mass. More severe lesions show deeper myofibrillar and focal cellular necrosis, spongiosis or intracellular oedema and sloughing of the epidermis (Copland and Willoughby, 1982), allowing hyphae to penetrate the basement membrane (Bootsma, 1973).

In 1877, an epizootic infection known as 'Salmon disease' occurred in wild salmon from rivers in England and Scotland, although a report of the Royal Commission on the Salmon Disease in England and Scotland (1877–1879), published by Buckland *et al*. (1880), failed to reach any conclusion regarding the cause. Although some investigators attributed outbreaks to a single species of oomycete, namely *S. ferax* (Stirling, 1878, 1880, 1881; Huxley, 1882a,b), others considered that the oomycete was a secondary infection and that the primary cause of the disease was bacterial in nature (Rutherford, 1881; Hume Patterson, 1903). Retrospectively, the 'Salmon disease' epizootic was clearly the first recognized case of ulcerative dermal necrosis (UDN) of salmonids (Roberts, 1972; Murphy, 1973).

Later, UDN was noted in Atlantic salmon (*Salmo salar*) in the Republic of Ireland, with epizootics subsequently occurring in Great Britain (Munro, 1970) and Europe (de Kinkelin and Le Turdu, 1971). The condition is unlike infections involving *Saprolegnia*, in that lesions occur on unscaled areas of the body, primarily the head. These lesions develop from small oval patches to become ulcerated, haemorrhagic and only during late stage development become infected with *Saprolegnia*. Some authors reported that the primary aetiology was viral (Roberts, 1972; O'Brien, 1974); however, there was some evidence for the role played by *Saprolegnia* from studies by Dunne (1970) and Roberts *et al*. (1971), who noted that these early lesions would heal if treated with malachite green.

Most of the literature on oomycete infections relates to teleost fish, in particular salmonids (Coker, 1923; Willoughby, 1985; Noga and Dykstra, 1986), and forms the predominant part of this review. In the past, the term 'fungal' was often used to represent fungal-like Chromalveolates or flagellated water moulds. However, nowadays, we try to avoid using the term 'fungus' in relation to water moulds and instead the term 'oomycete' is preferred.

The Disease Agent

Taxonomic position

Recent molecular studies have confirmed that oomycetes belong to the Kingdom Chromista, which forms part of the Chromalveolate

'super-kingdom' (Gunderson *et al.*, 1987; Baldauf *et al.*, 2000; Harper *et al.*, 2005; Cavalier-Smith and Chao, 2006). This lineage includes the chlorophyll-c containing algae, apicocomplexan parasites and ciliates. There continues to be some debate as to the Kingdom assignment for the heterokont lineage to which the oomycetes belong and, depending on the source, they may be placed both in the Kingdom Stramenopila (see Adl *et al.*, 2005) or the etymologically correct (but less widely used) Straminipila (Dick, 2001). In a recent analysis of heterokont flagellate groups, the oomycetes were placed with the anteriorly flagellate hyphochytrids and their sister clade, the parasitoid *Pirsonia*, and the free-living marine zooflagellate, *Developayella* (Tong, 1995), in a new phylum, the Pseudofungi (Cavalier-Smith and Chao, 2006). This group forms a sister clade to the photosynthetic algae, the Ochrophyta. However, there has been a reluctance to adopt the Pseudofungal phylum name and many textbooks continue to list the oomycetes as part of the phylum Heterokonta (Dick, 2001), or even as a separate phylum in their own right. Dick (2001) also proposed renaming the oomycete class the Peronosporomycetes, but again this new name has not been widely adopted and it seems the term 'oomycete' will continue to be retained for this class.

The class Oomycetes comprises around 800 species and traditionally is grouped into four orders, namely Lagenidiales, Leptomitales, Peronosporales and Saprolegniales (Sparrow, 1960). However, in his final taxonomic synthesis of the oomycetes in *Straminipilous Fungi*, Dick (2001) increased the number of orders to around 12 and formally proposed two major suborders, the Saprolegniomycetidae and Peronosporomycetidae, following on from the galaxy concept proposed by Sparrow (1976). Although these major subdivisions are supported by ultrastructural, biochemical and molecular data, it is clear from subsequent studies that many of the new orders and taxonomic rearrangements outlined by Dick (2001) are not supported by other scientists and the class will have to undergo further taxonomic studies (see Beakes and Sekimoto, 2009).

A list of oomycetes that are parasitic on live fish is summarized in Table 18.1. This includes isolations from living fish from either natural infections or experimental challenges. It is clear that most species are in the Saprolegniales, with only single representatives from each of the orders Leptomitales, Lagenidiales and Peronosporales, which were probably isolated as opportunists. The most prominent of these genera, with respect to the number of species and the frequency of their isolation, are *Aphanomyces* and *Saprolegnia*. Consequently, most of the following discussion concentrates on species from these two genera.

Historically, the identification and classification of *Saprolegnia* and other oomycetes have been a problem and the taxonomic status of many of the familiar taxa is problematic and continues to be so, even after the application of molecular techniques (see Hulvey *et al.*, 2007). This is because the delineation of genera in the Saprolegniales has been based largely on the morphology of their asexual zoosporangia and the way in which they produce spores (see Dick, 2001; Johnson *et al.*, 2002). Species within the genus *Saprolegnia* have been based largely on the morphology of the sexual reproductive structures (Dick, 1973, 2001; Willoughby, 1978; Johnson *et al.*, 2002). Unfortunately, many isolates of *Saprolegnia* taken from fish do not develop the sexual stages when cultured *in vitro* (Beakes and Ford, 1983; Grandes *et al.*, 2000; Diéguez-Uribeondo *et al.*, 2007).

Therefore, one of the problems with identifying isolates of water mould that are genuinely pathogenic to animals is that the name *S. parasitica* has been used in two different senses: firstly, as a repository for any asexual *Saprolegnia* isolate taken from fish or fish eggs and, secondly, to describe those sexual isolates which fit the descriptions of Kanouse (1932) and Coker and Matthews (1937) based on their oogonial characteristics as well as their ecology. It is these different uses that have led to much confusion and inaccuracy. In an attempt to solve these problems, Neish (1976) proposed that the name *S. parasitica* should be rejected, due to its ambiguity. The author argued that the

Table 18.1. Oomycete species reported as infections of live fish (updated from Neish and Hughes, 1980).

Fungal species	Reference
Order Saprolegniales, Family Saprolegniaceae	
Achlya spp.	Tiffney (1939a), Vishniac and Nigrelli (1957), Willoughby (1970), Bhargava *et al.* (1971), Nolard-Tintigner (1973, 1974), Srivastava (1976), Pickering and Willoughby (1977), Jha *et al.* (1977), Olah and Farkas (1978), Lartzeva and Dudka (1990), Sati (1991)
Achlya ambisexualis Raper	Vishniac and Nigrelli (1957), Willoughby (1970), Nolard-Tintigner (1973, 1974)
Achlya americana Humphrey	Scott and O'Bier (1962), Scott and Warren (1964), Ogbonna (1989), Sati (1991), Sati *et al.* (2009)
Achlya apiculata de Bary	Ogbonna (1989)
Achlya bisexualis Coker and Crouch	Vishniac and Nigrelli (1957), O'Bier (1960), Scott and O'Bier (1962), Jha *et al.* (1984), Lartzeva and Dudka (1990)
Achlya caroliniana Coker	Srivastava (1976), Srivastava and Srivastava (1977a), Ogbonna (1989)
Achlya diffusa Harvey	Srivastava (1976), Ogbonna (1989)
Achlya dubia Coker	Bhargava *et al.* (1971), Srivastava (1976), Ogbonna (1989)
Achlya flagellata Coker	Tiffney and Wolf (1937), Tiffney (1939a), Domashova (1971), Srivastava (1976), Ogbonna (1989), Lartzeva and Dudka (1990), Sati (1991)
Achlya hypogyna Coker and Pemberton	Ogbonna (1989), Lartzeva and Dudka (1990)
Achlya intricata Beneke	Howard *et al.* (1970)
Achlya klebsiana Pieters	Vishniac and Nigrelli (1957), Ogbonna (1989), Sati (1991)
Achlya megasperma Humphrey	Ogbonna (1989)
Achlya oblongata de Bary	Ogbonna (1989)
Achlya orion Coker and Couch	Srivastava (1976), Ogbonna (1989), Sati (1991)
Achlya prolifera Nees von Esenbeck	Nolard-Tintigner (1974), Srivastava (1976), Srivastava and Srivastava (1977b), Ogbonna (1989), Sati (1991)
Achlya proliferoides Coker	Srivastava (1976), Ogbonna (1989)
Achlya racemosa Hildebrand	Hoshina *et al.* (1960), Ogbonna (1989)
Achlya sparrowii Reischer	Vishniac and Nigrelli (1957)
Aphanomyces spp.	Shanor and Saslow (1944), Willoughby (1970), Srivastava (1976), Pickering and Willoughby (1977), Dykstra *et al.* (1986), Sati (1991)
Aphanomyces laevis de Bary	Vishniac and Nigrelli (1957), Srivastava (1976), Chien (1980), Ogbonna (1989), Lartzeva and Dudka (1990), Sati (1991)
Aphanomyces piscida	Miyazaki and Egusa (1972, 1973), Hatai *et al.* (1984)
Aphanomyces stellatus de Bary	Hoshina *et al.* (1960), Ogbonna (1989)
Calyptralegnia aclyoides (Coker and Couch) Coker	Vishniac and Nigrelli (1957)
Dictyuchus sp.	Norlard-Tintigner (1974), Tiffney (1939a), Sati (2009)
Dictyuchus anomalus Nagai	Srivastava (1976), Srivastava and Srivastava (1977c)
Dictyuchus monosporus Leitgeb	Norlard-Tintigner (1974), Lartzeva and Dudka (1990)
Dictyuchus sterile Coker	Srivastava (1976), Ogbonna (1989), Sati (1991)
Isoachlya anisospora var. *indica* Saksena and Bhargava	Srivastava (1976)
Isoachlya monilifera de Bary	Vishniac and Nigrelli (1957)
Isoachlya toruloides Kauffman and Coker	Ogbonna (1989)
Isoachlya unispora Coker and Couch	Domashova (1971), Ogbonna (1989)
Leptolegnia caudata de Bary	Willoughby (1970)

(*Continued*)

Table 18.1. *Continued*

Fungal species	Reference
Protoachlya paradoxa Coker	Vishniac and Nigrelli (1957), Sati (1991)
Pythiopsis sp.	Pickering and Willoughby (1977)
Saprolegnia spp.	Huxley (1882a,b), Johnston (1917), Coker (1923), Tiffney (1939a,b), Chidambaram (1942), Chaudhuri *et al*. (1947), Aleem *et al*. (1953), Lennon (1954), Vishniac and Nigrelli (1957), Arasaki *et al*. (1958), O'Bier (1960), Scott and O'Bier (1962), Scott and Warren (1964), Stuart and Fuller (1968), Bhargava *et al*. (1971), Norlard-Tintigner (1971, 1973), Bootsma (1973), Johnson (1974), Srivastava (1976), Neish (1976, 1977), Hatai *et al*. (1977), Pickering and Willoughby (1977), Sirikan (1981), Copland and Willoughby (1982), Gajdůšek and Rubcov (1985), Glazebrook and Campbell (1987), Oldewage and van As (1987), Okaeme *et al*. (1988), Pickering and Pottinger (1988), Lartzeva and Dudka (1990), Pohl-Branscheid and Holtz (1990), Sati (1991), Bly *et al*. (1992), Mohanta and Patra (1992), Das and Das (1993)
Saprolegnia australis Elliot	Hatai *et al*. (1977), Pickering and Willoughby (1977), Papatheodorou (1981)
Saprolegnia branchydanis	Ke *et al*. (2009)
Saprolegnia delica Coker	Vishniac and Nigrelli (1957), O'Bier (1960), Scott and O'Bier (1962), Dudka and Florinskaya (1971), Norlard-Tintigner (1973)
Saprolegnia diclina Humphrey	McKay (1967), Willoughby (1968, 1969, 1970, 1971, 1972, 1978), Norlard-Tintigner (1970, 1973), Srivastava (1976), Miyazaki *et al*. (1977), Hatai and Egusa (1977), Chien (1980), Smith *et al*. (1985), Bruno and Stamps (1987), Ogbonna (1989), Lartzeva and Dudka (1990), Sati (1991)
Saprolegnia ferax Thuret	Tiffney (1939a), Vishniac and Nigrelli (1957), O'Bier (1960), Hoshina *et al*. (1960), Scott and O'Bier (1962), Norlard-Tintigner (1970, 1971, 1973), Bhargava *et al*. (1971), Srivastava (1976), Srivastava and Srivastava (1977c), Smith *et al*. (1985), Ogbonna (1989), Lartzeva and Dudka (1990), Sati (1991), Le *et al*. (2009)
Saprolegnia hypogyna Pringsheim	Ogbonna (1989)
Saprolegnia litotalis Coker	Ogbonna (1989)
Saprolegnia invaderis Davis and Lazar	Davis and Lazar (1941)
Saprolegnia megasperma Coker	Vishniac and Nigrelli (1957)
Saprolegnia mixta de Bary	Vishniac and Nigrelli (1957), Dudka and Florinskaya (1971), Lartzeva and Dudka (1990), Sati (1991)
Saprolegnia monica Pringsheim	O'Bier (1960), Scott and O'Bier (1962), Domashova (1971), Sati (1991)
Saprolegnia parasitica Coker emend. Kanouse	Rucker (1944), Hoshina *et al*. (1960), O'Bier (1960), Scott and O'Bier (1962), Norlard-Tintigner (1973), Srivastava (1976), Jha *et al*. (1984), Toor *et al*. (1983), Ogbonna (1989), Krishna *et al*. (1990), Lartzeva and Dudka (1990), Sati (1991), Hatai and Hoshiai (1992, 1993), Yuasa and Hatai (1995a)
Saprolegnia shikotsuensis Hatai, Egusa, Awakura	Hatai *et al*. (1977)

Table 18.1.

Fungal species	Reference
Saprolegnia subterranea (Dissmann) Seymour	Pickering and Willoughby (1977)
Thraustotheca clavata (de Bary) Humphrey	Vishniac and Nigrelli (1957), Ogbonna (1989), Sati (1991)
Thraustotheca primoachlya Coker and Couch	Vishniac and Nigrelli (1957)
Order Leptomitales, Family Leptomitaceae	
Leptomitus lacteus (Roth) Agardh	Lennon (1954), Scott and O'Bier (1962), Willoughby (1970), Pickering and Willoughby (1977), Ogbonna (1989)
Order Peronosporales, Family Pythiaceae	
Pythium sp.	Scott and O'Bier (1962), Scott and Warren (1964), Sati (1991)
Pythium ultimum	Scott and O'Bier (1962)
Order Lagendiales, Family Lagendiaceae	
Lagenidium rabenhorsti Zopf	Kahls (1937)

Species names given are those used by the original authors. Several have synonyms and subsequently many have been reclassified. Any references to non-fruiting *Saprolegnia* isolates have been included under *Saprolegnia* spp.

criteria of oospore size and type used to differentiate between *S. parasitica* and other *Saprolegnia* isolates were of little diagnostic value and members of this species would be better assigned to *S. diclina* Humphrey. Willoughby (1978) considered the taxonomy of *S. parasitica* and *S. diclina* in detail and noted that there was sufficient similarity for all the isolates to be considered in the *S. parasitica–diclina* complex, and included *S. ashikotsuensis*, *S. australis* and *S. kauffmaniana*. Furthermore, Willoughby (1978) subdivided the *S. diclina* into three subspecific groups, based on oogonia morphology, with *S. diclina* type 1 infecting salmonid fish species and being synonymous with *S. parasitica* (Kanouse, 1932) and *S. diclina* (Willoughby, 1969, 1971, 1972; Pickering and Willoughby, 1977), type 2 occurring as a parasite of coarse fish and type 3 being entirely saprophytic (Hatai and Hoshiai, 1992). Since *S. diclina* can only be identified positively by its oogonium characteristics, it has meant that any sexually sterile isolates continue to be referred to simply as *Saprolegnia* spp. or, if taken from fish lesions, included with the *S. diclina* type 1 group.

However, this proposal overlooked the fact that a number of other independent characteristics, such as cyst coat morphology (Meier and Webster, 1954; Pickering *et al.*, 1979; Beakes, 1983) and differences in their esterase isozyme banding patterns (Beakes and Ford, 1983; Beakes *et al.*, 1994a) showed that type 1 isolates of '*S. diclina*' could always be separated from saprotrophic type 3 isolates (*S. diclina sensu stricto*). Many subsequent studies continued to try to apply the *S. diclina* type 1–3 terminology (Willoughby *et al.*, 1984; Wood and Willoughby, 1986; Wood *et al.*, 1988; Cross and Willoughby, 1989; Fregeneda-Grandes *et al.*, 2000, 2001). However, because of the distinctiveness of the fish-lesion isolates, many later authors reverted to calling both sexually sporulating and asexual fish-lesion isolates *S. parasitica* (Hatai and Willoughby, 1988; Hatai *et al.*, 1990; Willoughby and Roberts, 1992a; Beakes *et al.*, 1994a).

S. parasitica can also be distinguished by the fine structure of the secondary zoospore cyst cases (Fig. 18.3; see Pickering *et al.*, 1979; Hatai *et al.*, 1990; Söderhall *et al.*, 1991; Beakes *et al.*, 1994b) and a presumptive identification can be made using

phase-contrast microscopy (Willoughby, 1985). Studies using molecular markers have confirmed that *S. parasitica* is a genuine taxon (Fig. 18.4; Molina *et al.*, 1995; Whisler, 1996; Inaba and Tokumasu, 2002; Bangyeekum *et al.*, 2003; Diéguez-Uribeondo *et al.*, 2007). Recent studies have shown that isolates representing different geographical, morphological and pathological groups are part of the same genetically uniform clade, based on sequence analysis of the ITS-spacer region of the ribosomal DNA (Diéguez-Uribeondo *et al.*, 2007). This clade also encompasses other fish pathogenic taxa such as *S. salmonis*, which should be reduced to synonomy (Hussein and Hatai, 1999). With perhaps the exception of one or two isolates (Stueland *et al.*, 2005), all fish-lesion isolates with secondary cysts decorated by bundles of boathook hairs are part of this single genetic clade and form the taxon recognized as *S. parasitica*. *S. hypogyna*, which has secondary cysts decorated by single, rather than clustered, boathook spines (Hatai *et al.*, 1990), is the sister taxon (Fig 18.4). It should be noted that this clade is separate from that which includes the saprotrophic species, *S. diclina*, with which *S. parasitica* has often been considered to be conspecific using traditional oogonial characteristics (Willoughby, 1978). However, it is clear this confusion in nomenclature has also meant that not all molecular sequences for fish-lesion isolates have been entered in Genbank as *S. parasitica* (Diéguez-Uribeondo *et al.*, 2007). It is our hope that in the near future this taxon will be formalized correctly and a properly characterized lectotype culture identified.

The Saprolegniales form a well-supported clade, which diverges after the Leptomitales and as part of the larger Saprolegniomycetidae suborder (Dick, 2001). The large genus *Achlya* appears to be polyphyletic, the latter forming two or three subgroups (Inaba and Tokumasu, 2002; Spencer *et al.*, 2002). *Aphanomyces* spp. constitute the basal branch to the Saprolegniales clade (Fig. 18.4). *A. astaci* (Huang *et al.*, 1994; Lilley *et al.*, 2003) and *A. invadans* (Lilley

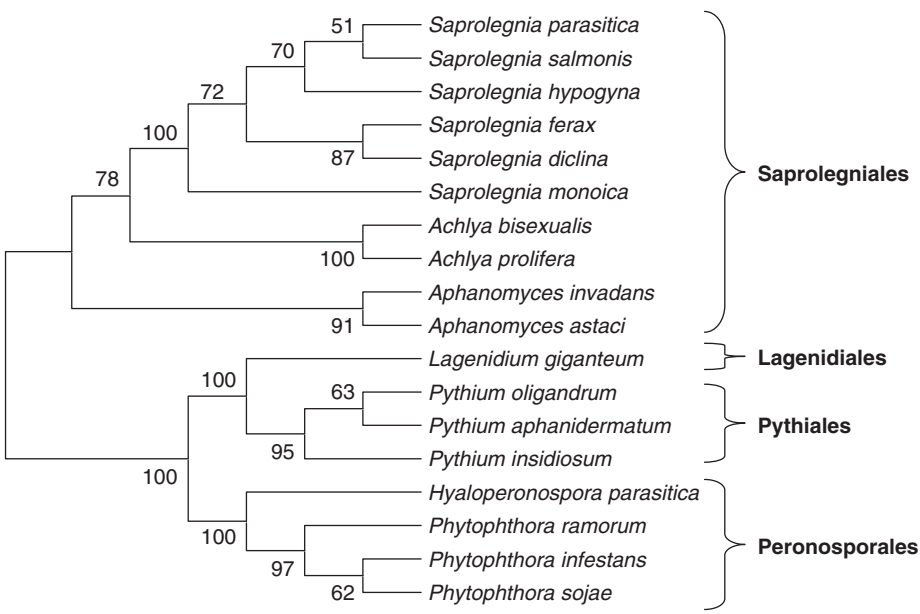


Fig. 18.4. Phylogenetic relationships between members of the Oomycetes. The phylogentic tree was constructed using the maximum parsimony methods based on ITS2 sequences and rooted at midpoint. Percentile bootstrap values based on 1000 replications are indicated at the nodes.

et al., 2003; Vandersea *et al.*, 2006) also form well-defined monophyletic species clades. A recent study on the molecular systematics of *Aphanomyces* has also shown that *A. invadans* and *A. piscicida* are genetically identical and these species should be reduced to synonomy (Diéguez-Uribeondo *et al.*, 2009).

Saprolegniasis

General and clinical signs

Saprolegniasis is frequently a superficial and chronic infection, with the appearance of cotton wool-like tufts on eggs or the integument and gills of fish (Neish and Hughes, 1980; Hussein *et al.*, 2001), which may extend over the entire body surface (Richards and Pickering, 1978) (Fig. 18.1). In severe cases, 80% of the body may be covered. In early infections, skin lesions are grey or white in colour, with a characteristic circular or crescent shape (Willoughby, 1989), which can develop rapidly and cause destruction of the epidermis. As infection develops, lethargy and loss of equilibrium follow, making the fish more susceptible to predation. The actual cause of death is likely to be associated with impaired osmoregulation (Gardner, 1974; Hargens and Perez, 1975). Respiratory difficulties may also feature when infection is associated with the gills (Bruno and Stamps, 1987). Pathogenic members of the Saprolegniaceae may penetrate major organs (Bootsma, 1973; Nolard-Tintigner, 1973, 1974; Dukes, 1975; Wolke, 1975; Hatai and Egusa, 1977) and the terms 'progressive dermatomycosis' or 'mycotic dermatomycosis' have been proposed (Hatai, 1980; Wada *et al.*, 1993).

Lesions normally do not appear at random, but are localized initially in specific areas associated with a physical insult, a concurrent infection with another pathogen (Neish and Hughes, 1980) or sexual differences of the host (White, 1975; Richards and Pickering, 1978). The latter seem attributable to differences in the number of goblet cells in the skin of male and female fish (McKay, 1967; Neish, 1977). However, the initial site of infection may not be confined to the skin or gills. Staff's disease in carp, for example, is saprolegniasis of the olfactory pits (Bauer *et al.*, 1973), infecting parts of the body such as the lateral line and cornea (Leibovitz and Pinello, 1980). Outbreaks of *S. ferax* involving the gut epithelium were reported in brook trout (*Salvelinus fontinalis*) (Agersborg, 1933) and rainbow trout fingerlings (Davis and Lazar, 1941), and the alimentary tracts of rainbow trout and amago salmon (*Oncorhynchus rhodurus*) fry were sites of infection for *S. diclina* (Hatai and Egusa, 1977; Miyazaki *et al.*, 1977).

Members of the genus *Saprolegnia* can be considered opportunistic facultative parasites, which are both saprotrophic and necrotrophic (Cooke, 1977). Peduzzi and Bizzozero (1977) suggested isolates of the *S. parasitica–S. diclina* complex and *S. ferax* were capable of progressing from saprotrophs to necrotrophs, due to their possessing a proteolytic enzyme, which resembled chymotrypsin. However, there is evidence that some Saprolegniaceae act as primary pathogens (Neish, 1977; Willoughby and Pickering, 1977; Willoughby, 1978; Noga *et al.*, 1988). Many workers have infected fish with *Saprolegnia* successfully under experimental conditions (Tiffney, 1939a; Vishniac and Nigrelli, 1957; Hoshina *et al.*, 1960; Scott and Warren, 1964; Nolard-Tintigner, 1970, 1971, 1973, 1974; Srivastava and Srivastava, 1977a,b,c), but it is questionable whether this is a cause of primary infection.

Predisposing factors

Fish are exposed continually to potentially pathogenic oomycetes and it therefore follows that a change in some predisposing factor or factors is necessary for infection to occur. Salmonids are susceptible to saprolegniasis throughout the freshwater stage of their life cycle, particularly leading up to and during smoltification (Pickering, 1994). Although *S. parasitica* can survive low salinity (Langvad, 1994), it cannot withstand

full salinity seawater and therefore the infection is absent from the marine phase in anadromous salmonid hosts. Saprolegniasis also shows a distinct seasonality, and this varies with the species of *Saprolegnia*. For example, *S. diclina* infections are common in winter months (Hughes, 1962), whereas *S. ferax* occurs predominantly in the spring and autumn (Coker, 1923; Hughes, 1962; Klich and Tiffney, 1985).

Role of sexual maturation

The association of *Saprolegnia* infection with sexual maturation and a similar increase in susceptibility to some common skin parasites, e.g. *Ichthyophthirius* and *Trichodina*, are documented (Pickering and Christie, 1980). Sexual maturation in brown trout (*S. trutta*) is accompanied by dramatic changes in epidermal structure and a decrease in mucus cells at the end of the spawning period (Pickering, 1977; Pickering and Richards, 1980), which is considered to exacerbate their susceptibility (Noga, 1993a). Although precocious mature male Atlantic salmon parr are susceptible to saprolegniasis, they also have an increased number of mucus cells (Murphy, 1981). However, no decrease in mucus cells during the prespawning period occurs, even with marked differences in susceptibility to oomycete infections (Pickering and Christie, 1980). The retention of zoospores of *S. diclina* on the epithelium of rainbow trout was also enhanced when experimentally challenged fish had been previously implanted with the androgen 11-ketotestosterone (Cross and Willoughby, 1989). Interestingly, the gross crescentic patterns of oomycete growth reflected those seen previously on wild salmonids only. Cross and Willoughby (1989) postulated that viable hyphae persisted only at the circumference of the advancing colony and this form of growth created the characteristic pattern.

Richards and Pickering (1978) noted that lesions were common on the dorsum in mature males and on the caudal fin in mature females. The activation of the pituitary–interrenal axis in teleosts is recognized as an almost ubiquitous component of the response to many different factors, most of which are considered stress related (Pickering, 1981). An increase in circulating corticosteroids has been used to assess the importance of the stress response in fish with *Saprolegnia* infection (Pickering and Duston, 1983). Prolonged oral administration of cortisol or natural increases in this hormone resulted in a marked increase in the susceptibility of the fish to infection. However, their plasma cortisol levels were within the levels capable of being produced by fish under natural stress (Pickering and Pottinger, 1985). Several authors have reported the immunosuppressive role of raised cortisol in salmonids (Pickering and Pottinger, 1985; Bennett and Wolke, 1987). A chronic increase in corticosteroid levels in brown trout from 1–4 ng/ml to 9–10 ng/ml increased the susceptibility of the fish to *Saprolegnia* infection (Pickering and Pottinger, 1985; Pottinger and Day, 1999). Observed changes may be due to infection in salmonids being associated with an increase in cortisol and certain sex hormones, and therefore an increase in susceptibility to saprolegniasis (Pickering, 1977). Brown trout parr treated with the chemosterilant methallibure during the later stages of spermatogenesis showed that hyperplastic changes in the epidermis were prevented and the prevalence of saprolegniasis reduced (Pottinger and Pickering, 1985). A similar finding was noted by Murphy (1981) with precocious male 1+ Atlantic salmon parr.

Integument integrity

Increased susceptibility to Saprolegniaceae from damage to the epidermis has been shown in fish under experimental conditions (Tiffney and Wolf, 1937; Tiffney, 1939b; Hoshina and Ookubo, 1956; Vishniac and Nigrelli, 1957; O'Bier, 1960; Scott and Warren, 1964; Srivastava and Srivastava, 1977a,b; Hatai and Hoshiai, 1994; Stueland *et al.*, 2005). Mechanical damage

from high stocking densities of farmed brown trout was considered responsible for an increased incidence of *Saprolegnia* infection (Richards and Pickering, 1978). The association of sexual maturity with the elevated occurrence of infection may, in part, be attributable to tegument damage sustained during spawning (Richards and Pickering, 1978).

Concurrent infection

Many species of *Saprolegnia* are secondary invaders, and infection with a primary pathogen renders the host more susceptible to opportunists. The condition UDN is a classic example where the disease is characterized by secondary *Saprolegnia* infection following an initial infection (Stuart and Fuller, 1968; Willoughby, 1968; O'Brien, 1974).

Primary bacterial infection associated with *Saprolegnia* sp. has been recorded in the Japanese eel (*Anguilla japonica*) (Hoshina and Ookubo, 1956; Egusa, 1965; Egusa and Nishikawa, 1965). Concurrent infestations with *Saprolegnia* sp. were observed in wild Atlantic salmon infected with *Gyrodactylus salaris* (Johnsen, 1978) and *Gyrodactylus* sp. (Heggberget and Johnsen, 1982), which damaged the skin of the host.

Environmental stress

Environmental stress factors, including poor water quality, adverse water temperatures and, in aquaculture, handling or overcrowding, can all result in increased occurrences of oomycete infections (Bailey, 1984). Annual outbreaks of saprolegniasis in wild brown trout were partially the result of an increase in organic debris in the water and a decreased flow rate (White, 1975). High organic loadings were identified as a cause of increased infection by *S. parasitica* (Toor *et al.*, 1983). Furthermore, rainbow trout exposed to sublethal levels of ammonia and nitrite increased their susceptibility to experimental infection with *S. parasitica* (Carballo and Muñoz, 1991). Social aggression in rainbow trout can increase susceptibility to this oomycete (Cross and Willoughby, 1989).

Pathogen Biology

Primary zoospores and primary cysts

The Saprolegniaceae are filamentous, coenocytic organisms producing a profusely branching aseptate vegetative mycelium. Unicellular, biflagellate zoospores are produced in sporangia, which are usually terminal and separated from the hyphal filaments by basal septa (Fig 18.2). The zoospores of *Saprolegnia* are diplanetic and dimorphic, comprising a pyriform primary and reniform secondary form, each with a different point of flagellum insertion – apical and lateral, respectively (Fig 18.3; Burr and Beakes, 1994). Primary zoospores are poor swimmers, serving merely to achieve a minimal level of dispersal from the parent colony on release from the zoosporangia. On settlement, zoospores encyst to form thin-walled cysts, often called encysted zoospores or simply cystospores. In many experimental studies on these organisms, it is unclear which asexual spore stage is being used in challenges and often all asexual spores are simply referred to as zoospores. Primary cysts are covered in tufts or single unbranched tubular hairs, which range in length from 0.5 μm for saprotrophic species to up to 1.5 μm in length for fish-lesion isolates (Pickering *et al.*, 1979; Beakes, 1983). In genera such as *Aphanomyces* and *Achlya*, the free-swimming primary zoospore phase is suppressed and balls of completely smooth primary cysts accumulate close to the sporangial exit pore (Fig. 18.3).

Secondary zoospores and secondary cysts

Primary cysts germinate and release the secondary zoospore, the main motile stage, which remains active for extended periods

until encystment occurs on a suitable substrate or host. It is assumed that the secondary zoospore is an important dispersive phase of the life cycle. Pickering and Willoughby (1982b) suggested that these spores might be chemotrophic, thereby enhancing the probability of pathogenic strains locating a fish host, although Smith *et al.* (1985) found that zoospores of pathogenic strains of *Saprolegnia* spp. were not attracted to live fish eggs. In contrast, Rand and Munden (1993b) demonstrated that extracts prepared from the chorionic membrane of live brook trout eggs induced a strong chemotactic response in zoospores of *S. diclina*, but this might be a characteristic of opportunist rather than pathogenic strains. Willoughby *et al.* (1983) demonstrated differences between species of *Saprolegnia* in their ability to encyst under different environmental conditions. They discovered that pathogenic isolates encysted more readily in sterilized lake water, which led Pickering and Willoughby (1982b) to propose that such isolates required a lower level of nutrient to stimulate encystment. Pickering and Willoughby (1982b) demonstrated that fish mucus stimulated encystment of zoospores from pathogenic isolates. Beakes *et al.* (1994a) reported other factors that induced encystment of *S. diclina* zoospores, including vortexing and incubation in dilutions of bovine serum albumin, haemoglobin and hemp-seed extract. The finely set encystment triggers in *S. parasitica sensu lato* suggest that the normal infective spore is probably the secondary cyst and attachment to the fish surface is probably a passive process (Wood *et al.*, 1988).

The secondary cysts are decorated by bundles (of between 5–20 spines) of long, hooked spines, which may vary in length (*c.*2.5–14 μm). Single, short boathook hairs were first described by Manton *et al.* (1951) in an isolate of *S. ferax* and, shortly afterwards, Meier and Webster (1954) noted that the secondary cysts of *S. parasitica* were decorated by bundles of much longer spines, but with the same boathook morphology, and this was confirmed in many other studies (Pickering *et al.*, 1979; Hallett and Dick, 1986; Puckridge *et al.*, 1989; Hatai *et al.*, 1990). There appears to be a linear correlation both for the number of spines in a bundle (between *c.*4 and 16) and overall spine length (Pickering *et al.*, 1979; Beakes *et al.*, 1994a) and between the mean length of hairs on the primary and secondary cysts (Beakes, 1983). An analysis by Fregeneda-Grandes *et al.* (2000) of secondary zoospore cyst formation of *Saprolegnia* from infected wild brown trout indicated the presence of two distinct morphotypes among long-spined isolates. The isolates with a higher number of bundles per cyst and bundles with a greater number and length of hairs were included in cyst morphological Group I, and those with a smaller number and shorter length of these bundles and hairs were included in cyst morphological Group II. What was interesting was that Group II isolates were all from salmonids with saprolegniasis, whereas most isolates from mucus and water belonged to Group I. So, while the presence of bundles of spines over 2 μm in length on the secondary cysts are reliable markers for *S. parasitica* as a taxon, their size and number do not appear to be an indicator of aggressiveness and their role in infection is therefore still unclear. Until genetically modified pathogenic isolates without spines can be generated and tested, then a question mark has to be placed over their role as determinants of pathogenicity.

The surface of both primary and secondary zoospores has a glucomannan glycocalyx, which binds concanavalin A (Lehnen and Powell, 1993; Burr and Beakes, 1994). The primary and secondary cyst surface also binds this lectin, although in secondary cysts labelling may be patchy and vary in intensity (Burr and Beakes, 1994). In thin-sectioned material, this lectin binding is associated with the outermost layer of the cyst wall and is also associated with a matrix material that coats both long and short boathook spines (Burr and Beakes, 1994). Both this matrix and the spines are formed in peripheral encystment vesicles, formerly known as bar-bodies due to their characteristic shape in *S. ferax* (Heath and Greenwood, 1970), that are found throughout

the Saprolegniaceae (Beakes, 1983, 1989, 1994; Randolph and Powell, 1992). In addition, at the time of encystment, the larger kinetosome-associated vesicles (K2 bodies) release a pad of adhesive material (Lehnen and Powell, 1991; Durso *et al.*, 1993; Burr and Beakes, 1994), which binds the lectin wheatgerm agglutinin (WGA) (Powell *et al.*, 1985; Burr and Beakes, 1994) and contains catalase (Powell *et al.*, 1985). This material is thought to play a significant role in attachment of the zoospore to a specific substrate, particularly in saprotrophic species (Lehnen and Powell, 1989; Beakes *et al.*, 1994a) and probably has little role in attachment to fish.

Secondary zoospores are capable of polyplanetism, by which repeated cycles of encystment and release are undergone if an appropriate substrate is not found initially (Diéguez-Uribeondo *et al.*, 1994). This behaviour is particularly common in isolates of *S. parasitica* and is thought to be an important adaptive feature of the life cycle, which enables propagules to make several attempts at locating appropriate substrates (Beakes *et al.*, 1994a; Diéguez-Uribeondo *et al.*, 1994). The actual stimuli for encystment and germination remain uncertain, but the phenomenon of polyplanetism suggests they are different. The expression of spore-specific transcripts at different stages of the asexual life cycle of *S. parasitica* was examined by Andersson and Cerenius (2002). They reported that one of the markers, designated *puf1*, was found to be expressed transiently upon each of several cycles of zoospore encystment and re-emergence. The transcript is induced immediately upon zoospore encystment and is lost rapidly when a cyst is triggered to germinate. In non-germinating cysts, *puf1* is maintained until a time point when the cysts can no longer be triggered to germinate and thus have become determined for zoospore re-emergence. The results show that the cyst stage has two phases, of about equal duration, which are physiologically and transcriptionally distinct and that the transcriptional machinery of oomycetes is also active in non-germinating spores.

Germination and growth

Germination and growth of pathogenic species of *Saprolegnia* are rapid when compared with saprophytic members of the genus and may be determined by the nutrient levels of the supporting medium (Willoughby *et al.*, 1983). Saprophytic species, including *S. diclina* and *S. ferax*, normally were unable to germinate in sterilized lake water, whereas isolates from salmonid lesions, including *S. diclina* type 1 and *Saprolegnia* sp., germinated readily in the same medium (Willoughby *et al.*, 1983). The rate of germination of these strains was enhanced two- to fivefold in water taken from the effluent of a salmonid fish farm, whereas saprophytic isolates failed to germinate.

Germination has been described as either direct or indirect. Typically, germination is direct where cytoplasm-filled germ tubes are wide (*c.*8–10 μm) and coenocytic (Willoughby, 1977). In contrast, Willoughby *et al.* (1983) and Willoughby and Roberts (1992a) have shown that germination of parasitic *Saprolegnia* isolates, grown in either fish mucus or sterilized lake water, is indirect and characterized by a partially evacuated germination, whereby the germ tube is uniform, narrow (*c.*3–5 μm) and septate, with the cytoplasm being confined to the apical tip segment alone (Fig. 18.3). This pattern of growth may enable the rapid growth of pathogenic isolates (Willoughby *et al.*, 1983; Beakes *et al.*, 1994a). Pickering and Willoughby (1982b) proposed that the evacuated germling type could represent the infective agent, with the increased length facilitating attachment to the host. Pickering and Willoughby (1982b) speculated that the extended morphology of these germlings might enable increased nutrient uptake.

Once germination has occurred, the normal life cycle is sometimes abbreviated, with the production of an additional zoospore stage, in situations where nutrient availability is low (Willoughby, 1977). A single sporangium is formed, releasing one or two secondary zoospores that subsequently encyst in the normal way. This

phenomenon may represent a mechanism that ensures dispersal during periods when conditions are suitable for encystment and germination, but not growth.

Sexual reproduction

Many isolates of *S. parasitica* from fish do not reproduce sexually and species such as *A. invadans* are entirely asexual. In general, sexually reproducing Saprolegniaceae identified from fish hosts are homothallic, with both male and female sexual reproductive structures appearing on a single mycelium (Fig. 18.2). Sexual reproduction occurs by gametangia contact, resulting in the fusion of the haploid oosphere with sperm nuclei (Beakes and Gay, 1977). Resulting oospores have a central ooplast (Howard, 1971), which in the Saprolegniales usually have a granular appearance, and contain large quantities of lipids, thought to represent food reserves. Germination normally occurs following a resting period (Dick, 1972; Dick and Win-Tin, 1973). Sexually reproducing isolates of *S. parasitica* usually only reproduce sexually after 4–6 weeks at suboptimal temperatures (Willoughby, 1978). Typically, the oospores of this species are small, or of variable size, and very often there is evidence of premature oosphere abortion (Beakes and Ford, 1983).

Geographic Distribution and Host Range

Infection involving members of the oomycetes is extensive in both wild and farmed fish (Willoughby and Pickering, 1977; Neish and Hughes, 1980) and is considered ubiquitous in freshwater ecosystems (Neish and Hughes, 1980; Waterstrat, 1997). Many researchers have reported infections on salmonid species and their eggs and, in the UK, *S. parasitica sensu lato* (as defined above) has been isolated from Atlantic salmon (Willoughby, 1986), rainbow trout, brown trout and Arctic charr (*S. salvelinus*) (Pickering and Christie, 1980; Wood and Willoughby, 1986). Hatchery-reared and wild brown trout succumb to infection by *S. diclina* at spawning (Pickering and Christie, 1980), while increased losses of cultured rainbow trout fry have been associated with infection. For example, Bruno and Stamps (1987) reported losses of farmed Atlantic salmon first-feeding fry due to *S. diclina*. Saprolegniasis is a serious problem in Atlantic salmon hatcheries in Ireland (Smith, 1994) and in Norway (Langvad, 1994), where infections of *Saprolegnia* sp. occur in wild Atlantic salmon parr. In Japan, epizootics due to *S. parasitica* have occurred in farmed coho salmon (Hatai and Hoshiai, 1992, 1993). *S. parasitica* and *S. diclina* have also been implicated in mortality of cultured rainbow trout, coho salmon and ayu (*Plecoglossus altivelis*) in Japan (Yuasa and Hatai, 1995a). In Japan, a *Saprolegnia* epizootic involving eggs and cultured salmon was attributed to *S. parasitica*, *S. salmonis* (although these two species are almost certainly conspecific; Diéguez-Uribeondo *et al.*, 2007) and *S. australis* by Hussein *et al.* (2001). The infection of salmonids with *S. parasitica* has occurred in Australia (Puckridge *et al.*, 1989) and the USA (Mueller and Whisler, 1994). Furthermore, Chien (1980) discovered *S. diclina* and *A. laevis* in spawning rainbow trout in Taiwan, and found *Pythium debaryanum* saprophytic on dead eggs, although it is likely all of these are opportunists rather than aggressive pathogens.

Reports of oomycete occurring on non-salmonid fish have been noted in the UK, with isolations from the following fish: *Achlya*, *Aphanomyces*, *Leptolegnia*, *Leptomitus*, *Pythiopsis* and *Saprolegnia* from perch (*Perca fluviatilis*) (Willoughby, 1970; Pickering and Willoughby, 1977; Bucke *et al.*, 1979). Paxton and Willoughby (2000) reported that spores from adjacent dead eggs did not infect perch eggs, suggesting that these eggs had some anti-oomycete properties within the gelatinous mass. In France, mortality caused by *S. australis* in cultured roach (*Rutilus rutilus*) has occurred (Papatheodorou, 1981). Other 'coarse' fish can be hosts for *S. diclina* and they include orfe (*Leuciscus idus*), carp (*Cyprinus*

carpio), tench (*Tinca tinca*) and pike (*Esox lucius*) (Pickering and Willoughby, 1982b). These authors also isolated *Saprolegnia* from eels and the river lamprey (*Lampetra fluviatilis*). Elvers of *A. anguilla* cultured intensively in warmwater effluent suffered high mortality due to *Saprolegnia* sp. (Copland and Willoughby, 1982). Gajdûšek and Rubcov (1985) reported damage to wild carp eggs in the former USSR (Russia) caused by *Saprolegnia* sp. and, in the USA, Xu and Rogers (1991) isolated *Saprolegnia* from channel catfish (*Ictalurus punctatus*), which resulted in a massive mortality during the winter months (Bly *et al.*, 1992; Bangyeekhum *et al.*, 2001). Saprolegniasis also affects cultured Atlantic sturgeon (*Acipenser oxyrhynchus*) at spawning (Smith *et al.*, 1980) and cultured juvenile barramundi (*Lates calcarifer*) in Australia (Glazebrook and Campbell, 1987).

In Nigeria, Ogbonna (1989) showed that a wide range of oomycetes, including members of the genera *Achlya*, *Aphanomyces* and *Saprolegnia*, had been found in various freshwater hosts. Also in Nigeria, Okaeme *et al.* (1988) found the eyes of hatchery-reared tilapia (*Oreochromis niloticus*) infected with *Myxosoma sarigi* and with *Saprolegnia* sp. Fingerlings of another tilapia species (*O. mossambicus*), cultured in South Africa, were reported as common hosts for *Saprolegnia* sp. (Oldewage and van As, 1987) and El-Sharouny and Badran (1995) recovered *Achlya americana*, *A. dubia*, *A. flagellata*, *A. racemosa* and *S. ferax* and *S. diclina* from this fish. In Brazil, silver mullet (*Mugil curema*) reacclimatized in fresh water were susceptible to *Saprolegnia* sp. (Conroy *et al.*, 1986). Another species of mullet (*Liza abu*) and carp cultured in Iraq were infected with *A. polyandra*, *S. ferax* and *S. terrestris* (Butty *et al.*, 1989).

Saprolegniasis has been noted in cultured teleosts in Indonesia (Sirikan, 1981) and India (Srivastava, 1980; Sati, 1991). High mortality, attributed to *S. parasitica*, was recorded by Krishna *et al.* (1990) in various species of major carps cultured in ponds and in silver carp (*Hypophthalmichthys molitrix*) cultured in cages (Jha *et al.*, 1984). Furthermore, infection with this oomycete species was the cause of losses in carp and Indian major carp (*Labeo rohita*) during the winter when the water had a high organic loading (Toor *et al.*, 1983). Singhal *et al.* (1990) detected a significant suppression of growth in cultured carp in which *Saprolegnia* sp. was associated with an *Argulus indicus* infection. Air-breathing teleosts (*Anabas testudineus*) from a river in India were also found to carry *S. parasitica* infections (Mohanta and Patra, 1992). Freshwater tropical aquarium species commonly living in waters of 23°C have also shown clinical signs of saprolegniasis, and they include *Plecostomus* spp. (Leibovitz and Pinello, 1980) and the Mexican platyfish (*Xiphophorus maculatus*) (Vishniac and Nigrelli, 1957).

Epizootic ulcerative syndrome (EUS) is one of the most destructive fish diseases affecting farmed species in Asia and the Indo-Pacific region (Frerichs *et al.*, 1986; Hatai *et al.*, 1994; Devaraja *et al.*, 2004). The causative agent, *A. invadens* (now synonymous with *A. piscida* described by Hatai *et al.*, 1984), and the disease is also known as red-spot disease (Callinan, 1985; Callinan *et al.*, 1995; Lilley and Roberts, 1997; Lilley *et al.*, 2003; Johnson *et al.*, 2004) and mycotic granulomatoses (MG), and is a disease reportable to the Office International des Epizooties (OIE). EUS is a seasonal epizootic condition with a complex infectious aetiology which has occurred in over 100 species of wild, farmed freshwater and brackish water fish (Lilley *et al.*, 1998; OIE, 2006), primarily the Atlantic menhaden (*Brevoortia tyrannus*) in the north-western Atlantic Ocean (Noga and Dykstra, 1986; Blazer *et al.*, 2002; Kiryu *et al.*, 2003). The first report of EUS in farmed ayu (*P. altivelis*) occurred in Japan during 1971 (Egusa and Masuda, 1971) and it was reported later in grey mullet (*M. cephalus*) in eastern Australia in 1972 (McKenzie and Hall, 1976).

Several *Aphanomyces* spp. and *Saprolegnia* spp. were also associated originally with an ulcerative mycosis in the USA from a range of estuarine species, primarily the Atlantic menhaden in the north-western Atlantic Ocean (Dykstra *et al.*, 1986). Deep skin lesions are characteristic of the

infection, which eventually involves the internal organs and an intense inflammatory reaction (Dykstra *et al.*, 1986; Noga *et al.*, 1988). The causal agent was ascribed initially to a number of opportunistic *Aphanomyces* rather than the true pathogen, now known to be *A. invadans* (see Vandersea *et al.*, 2006). This is in contrast to the superficial lesions and mild inflammatory response normally observed in *Saprolegnia* infections of freshwater fish (Bly *et al.*, 1992; Álvarez *et al.*, 1995).

Recent studies have confirmed that the ulcerative mycosis of the Atlantic menhaden is also due to the same pathogen (Blazer *et al.*, 2002), suggesting that many of the oomycete isolates previously identified with this syndrome were opportunists rather than the primary pathogen. A similar type of infection has also been reported in mullet (*M. cephalus*) (Roberts *et al.*, 1986), although the causal agent was not cultured. EUS was first reported in farmed freshwater aya (*Plecoglossus altivelis*) in Oita Prefecture, Kyushu Island, Japan in 1971 (Egusa and Masuda, 1971). The disease then extended its range through Papua New Guinea into South-east and South Asia, and into West Asia, where it was reported in Pakistan (Tonguthai, 1985). EUS has now been reported from 24 countries in four continents: North America, Southern Africa, Asia and Australia (FAO, 2004). Outbreaks of an ulcerative disease have been reported in menhaden in the USA, and reported as similar to EUS in Asia (Dykstra *et al.*, 1986; Blazer *et al.*, 1999). Epizootic ulcerative syndrome is endemic in many freshwater catchments and estuaries in Australia and has been reported officially from New South Wales, Northern Territory, Queensland, Victoria and Western Australia (Callinan *et al.*, 1995). This may be a pathogen that has the potential to spread into Europe and cause significant problems.

Economic Importance of the Disease

Fish mycoses are considered difficult to prevent and treat, particularly in intensive freshwater systems, and are reported to be second only to bacterial disease in economic importance to aquaculture (Meyer, 1991). Water mould epizootics generally are rare in wild fish populations; however, during the 1960s, thousands of wild Atlantic salmon died during their migration into rivers in the British Isles and southern Ireland from ulcerative dermal necrosis (UDN) (see Roberts, 1993). Such outbreaks increased public awareness of losses in wild salmon stocks and consequently promoted an increase in fisheries research. However, despite a great deal of work, the cause of UDN has remained controversial (Stuart and Fuller, 1968) and to date has not been established (Carberry, 1968).

Outbreaks of *Saprolegnia* spp. in farmed fish usually are restricted to chronic but steady losses; however, there has been a dramatic resurgence of *Saprolegnia* infections in aquaculture since the use of the malachite green was banned (Torto-Alalibo *et al.*, 2005). *S. parasitica* has been identified from many fish and is considered a significant pathogen (Willoughby, 1969; van West, 2006). Egg mortality (Fig. 18.1) can increase rapidly, causing significant losses, which may have a major economic impact. During severe winters, some farmers in the USA have reported losses of up to 50% in farmed catfish, with an annual economic cost of about US$40 million (Bly *et al.*, 1994). *Saprolegnia* infection is believed to contribute significantly to overall losses in elver (*A. anguilla*) culture (Copland and Willoughby, 1982). In Japan, significant losses, exceeding 50% per annum, attributed almost exclusively to *S. parasitica*, have been reported in farmed coho salmon (*O. kisutch*) (Hatai and Hoshiai, 1992, 1993). Significant mortality from oomycete outbreaks has also occurred in wild and farmed brown trout in Spain (Aller *et al.*, 1987; Diéguez-Uribeondo *et al.*, 1996).

In the eastern USA, up to 80% of the commercial catch of Atlantic menhaden can be affected, and this ulcerative mycosis (EUS) has been attributed to an *Aphanomyces* infection during the unusually wet spring and summer of 1984, which created conditions of low salinity. These losses, with sporadic outbreaks in earlier years,

represented a severe threat to this industry, which, at the time, was worth US$27 million per annum (NCDMF, 1983).

Diagnostic Methods

Isolation

One primary difficulty encountered when investigating oomycete diseases of fish is isolating the pathogen. Many *Saprolegnia* infections occur in the dermis and therefore more than one species easily may occur in lesions at the same time (Pickering and Willoughby, 1982a,b). Many saprophytic species may be present and their growth in culture may be rapid, thereby masking the primary species. This is particularly true of older infections, where the number of secondary saprophytic organisms is likely to be high (Alderman, 1982). Consequently, sampling of dead animals should be avoided wherever possible (Willoughby, 1971). A squash preparation of a skin scrape from the lesion can be a useful preliminary screening method for fungi, oomycetes and ectoparasites (Pickering and Christie, 1980).

Initial isolation is usually made from small pieces of the affected tissue (approximately 5 mm^3). Hatai (1989) advised that deep-seated tissues should be used wherever possible, to limit contamination with bacteria or opportunists. All tissues should receive some form of pretreatment to remove any contaminant species. Several regimes have been employed to achieve this, including surface sterilization by immersion in ethanol, washing with sterile water and irradiation (Tiffney, 1939b). Glass rings were included by Raper (1937) to reduce bacterial contamination. Willoughby (1978) described a procedure whereby infected salmonid tissues were cut into small pieces and incubated in sterile lake water for up to 5 days at 7°C, observed regularly for different forms and a decision made on whether to make isolations from zoospores or hyphae. This method, although extremely thorough, is impractical as a routine diagnostic procedure. Instead, viable isolates can be extracted from the tissues, following washing, and cultured using an appropriate artificial medium (Willoughby and Pickering, 1977).

To avoid mixed culture growth, isolations should be carried out from single spores or hyphal tips of the initial culture wherever possible (Neish and Hughes, 1980; Alderman, 1982). Furthermore, these isolates should be subjected to infection experiments using the same host species to determine pathogenicity. In practice, however, this is rarely carried out and, for salmonids, it would appear that small numbers of isolations from affected fish are sufficient to determine the pathogenic role of the oomycete isolate (Neish, 1977; Willoughby, 1978).

Impression smears (Krishna *et al.*, 1990) or squashes (Pickering and Christie, 1980) are used to provide a presumptive diagnosis. These techniques are useful in providing an indication of the organism prior to cultivation and used for identification to genera. Imprints stained with a lactophenol blue (Krishna *et al.*, 1990) or methylene blue (Bruno and Stamps, 1987) are viewed by light microscopy. Squashes are usually observed using dark-field or phase-contrast microscopy.

Culture

A wide range of media has been used for the isolation and culture of fungi and oomycetes from fish, and antibiotics have been incorporated to help reduce bacterial contamination. However, in some circumstances, oomycetes may be too sensitive to permit the use of antibiotics, as with the slow-growing *A. astaci* and *A. invadans* (Unestam, 1965; Lilley and Inglis, 1997). Penicillin and streptomycin are the antibiotics most commonly used and are incorporated into appropriate media at 150–1000 iu/ml and 250–1000 mg/ml, respectively. Other antimicrobial agents, such as carbenicillin, chloramphenicol, gentamycin, neomycin, oxolinic acid and potassium tellurite, are used to a lesser extent. Agar plates are inoculated with pieces of infected

tissue, using aseptic technique. The plates are incubated and observed at frequent intervals for emerging hyphal tips (Noga and Dykstra, 1986) and then transferred to fresh media to obtain a single oomycete culture. Isolations by inoculating mycelium directly from fish or eggs on to an agar medium have been made (Neish, 1975; Willoughby and Pickering, 1977). Alderman (1982) suggested that bacterial contamination could be kept to a minimum by using a low nutrient medium incorporating antibiotics and flooding agar plates with a thin layer of fresh or seawater. Incubation temperatures range between 5 and 37°C; however, temperatures of 10, 15 and 20°C are most common.

For marine or estuarine species, media can be supplemented with salt, usually at a concentration up to 3% (Hatai, 1989), or prepared using sterile seawater (Fuller *et al.*, 1964; Bian *et al.*, 1979; Lightner, 1988). Stevens (1974) described a range of media devised specifically for oomycete isolation from the marine environment.

An alternative method to using agar plates is the more traditional system of 'baiting'. Using techniques described by Johnson (1956), Seymour (1970) and Stevens (1974), infected fish tissues, soil or water samples are incubated with hempseeds. The resulting oomycete colonies are transferred either to fresh hempseed media or to sterile water to obtain single strain cultures. Willoughby (1985) advocated the use of placing *Saprolegnia* infested hempseeds in water as a satisfactory and rapid method for *Saprolegnia* zoospores.

Wallpaper paste (Polycell) has been used as an alternative to traditional agar (Willoughby *et al.*, 1984), particularly for the culture of *Saprolegnia* from water samples (Willoughby, 1986; Wood and Willoughby, 1986) and fish (Singhal *et al.*, 1987). The paste seems to act as a good physical support for the oomycete, allowing separation of the growing colonies. Samples are mixed with nutrients and the paste in a Petri dish and incubated at 20°C for 24–72 h, after which time single colonies may be transferred to sterile water for identification of sporangia, zoospores and oogonia. An improvement in the recovery of oomycetes using hydroxyethyl cellulose (Natrosol 250) as a replacement for wallpaper paste was reported by Celio and Padgett (1989).

There are numerous methods available to preserve oomycetes. However, two methods widely used by culture collections to achieve successful preservation of plant pathogenic species are cryopreservation with liquid nitrogen using controlled-rate freezing and centrifugal freeze-drying (Smith and Ryan, 2008). However, these techniques have proved problematic with aquatic oomycetes.

Identification

Identification of oomycetes has relied largely on micromorphology and sporulation characteristics, the limitations of which already have been discussed above (Coker, 1923; Sparrow, 1960; Seymour, 1970; Willoughby, 1978). Yuasa and Hatai (1994) attempted to identify *Achlya*, *Aphanomyces* and *Saprolegnia* from their biological characteristics. They investigated optimal growth temperature and sensitivity to a range of chemicals and found differences between genera and their sensitivity to malachite green, sorbose and Polyphenon–100. They were also able to differentiate between a saprophytic and a pathogenic strain of *A. piscida*.

Initial indications suggest that developing an immunological assay to identify oomycete infections in clinical outbreaks may be feasible (Bullis *et al.*, 1990). Preliminary investigations using polyclonal (Bullis *et al.*, 1990) and monoclonal antibodies (Beakes *et al.*, 1994a; Burr and Beakes, 1994; Bullis *et al.*, 1996) raised against various epitopes of *Saprolegnia* indicate that developing a rapid antibody-based assay for the detection of oomycete infections of fish may be possible. Murine polyclonal antibodies to *S. parasitica* were also observed to cross-react with other *Saprolegnia* species, but with further investigation, these authors were confident a more specific assay could be developed. Unfortunately, cross-reaction

with all *Saprolegnia* genera was observed with the monoclonal antibodies raised against the same species (Burr and Beakes, 1994). These authors also found that the monoclonal antibody cross-reacted with other genera within the Saprolegniales, including *A. astaci* and *Achlya* sp.

An early PCR-based approach to examine ribosomal DNA from *Saprolegnia* isolates obtained from many geographical locations was developed by Molina *et al.* (1995). This group found identical DNA fingerprints from all strains of *S. parasitica* examined, and suggested that this could form the basis of a diagnostic test to be applied in the absence of morphological characters. Diéguez-Uribeondo *et al.* (1996) also developed a method employing RAPD, using PCR with DNA from *S. diclina–parasitica* complex isolates. They showed that Spanish isolates of this oomycete had a genetic similarity of 85–100%, compared with only a 20–45% similarity with other strains of the *S. diclina–parasitica* complex. Whisler (1996) noted that *S. parasitica* isolates from the Columbia River basin, USA, recognized by RAPD analysis, were representative of the vast majority of the isolates cultured from lesions. The use of single and paired primers with PCR amplification permitted identification of pathogenic groups and their distinction from other species of the genus considered more saprophytic in character (Whisler, 1996). The successful development of a PCR technique for the identification of the *S. diclina–parasitica* complex is well documented (Molina *et al.*, 1995; Diéguez-Uribeondo *et al.*, 1996, 2007; Bangyeekhum *et al.*, 2001, 2003). This method provides a sensitive and rapid assay for the assessment of genetic distance between different isolates. Bangyeekhum *et al.* (2003) used the RAPD-PCR technique and the presence or observation of repeated zoospore emergence to characterize *Saprolegnia* sp. isolates. They reported the majority of the isolates belonged to a single genetically defined group. More recently, Diéguez-Uribeondo *et al.* (2007) proposed that, based on molecular analysis and morphological strain typing, the use of the name *S. parasitica* should be assigned to isolates forming cysts with bundles of long-hooked hairs.

Monoclonal antibodies (MAbs) prepared against an *S. parasitica* isolate from brown trout have been used to detect and differentiate between isolates with bundles of long hairs (*S. parasitica*) and other *Saprolegnia* species through an indirect immunofluorescence assay (Fregeneda-Grandes *et al.*, 2007a). One MAb (18A6) was able to differentiate between the asexual and most of the sexual isolates in the group of long-haired *S. parasitica* isolates, but did not recognize *Achlya* sp. or the *Saprolegnia* species without bundles of hairs, with the exception of *S. hypogyna*. These results indicate that isolates with bundles of long hairs are related closely to other members of genus *Saprolegnia* and share several antigens. However, MAb 18A6 seems to recognize an epitope that is expressed mainly in the asexual isolates in the *S. parasitica* clade.

The current methods of diagnosis for EUS are based on clinical signs of EUS and confirmed by histopathological examination and recognition of mycotic granuloma (MG) with aseptate invasive hyphae. Briefly, scales are removed from the periphery of the lesion and a sterile scalpel blade and sterile fine-pointed forceps are used to cut and lift the stratum compactum to reflect superficial tissues, thereby exposing the underlying muscles. Moderate, pale, raised dermal lesions are most suitable for oomycete isolation. Affected muscles should be excised carefully and approximately 2 mm^3 placed on a Petri dish containing Czapek Dox agar with penicillin G (100 units/ml) and oxolinic acid (100 µg/ml). The plates are sealed and then incubated at room temperature and examined daily. Emerging hyphal tips are transferred repeatedly on to fresh plates of Czapek Dox agar until cultures are free of contamination (OIE, 2006).

A. invadans does not produce sexual structures and therefore cannot be diagnosed by morphological criteria alone. It should be noted that ongoing molecular studies of *Aphanomyces* spp. (Diéguez-Uribeondo *et al.*, 2009) have shown that the EUS causal agent, *A. invadans*, is conspecific with *A. piscida*, described earlier as

associated with a mycotic granulomatosis of ayu and other freshwater fish (Hatai *et al.*, 1984). However, this species can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics, as described by Lilley and Chinabut (2000). *A. invadans* is slow-growing in culture and fails to grow at 37°C on GPY agar (Table 18.1).

All known *A. invadans* isolates belong to a single genotype, which facilitates identification, and therefore this pathogen is identified readily by RAPD-PCR or sequencing of the internal spacer (ITS) region of nuclear rDNA. RAPD-PCR is rapid and does not require DNA sequencing, but first it is necessary to isolate the pathogen in pure culture for reliable results. The details regarding DNA preparation and PCR primers for amplification can be found in Lilley *et al.* (1997a) and Lilley and Chinabut (2000). Alternatively, sequencing of the internal spacer region can be performed and the results compared with the sequence deposited in public gene data banks.

Lilley *et al.* (2001) compared 21 isolates of *A. invadans* with other oomycetes in terms of their pyrolysis mass spectrometry (PyMS) profiles. Canonical variate analysis (CVA) of the pyrolysis mass spectra distinguished the *Aphanomyces* species from widely dispersed *Achlya* and *Saprolegnia* isolates. Furthermore, CVA and hierarchical cluster analysis (HCA) separated the *Aphanomyces* species into two main groups. The first group clustered *A. invadans* isolates from EUS outbreaks in Australia, Bangladesh, Indonesia, Japan, the Philippines and Thailand together. However, HCA also included *A. astaci* in this group. Non-pathogenic *Aphanomyces* strains isolated from ulcerative mycosis affected fish were shown to be distinct from *A. invadans* and instead clustered with saprophytic *Aphanomyces* strains to form the second group. Although, this is the first report using PyMS in the study of oomycete systematics, the technique was not considered sensitive to show any intraspecific differences, but considered as useful for the discrimination of species with uncertain taxonomic relationships.

Recently, Vandersea *et al.* (2006) developed a PCR and fluorescent peptide nucleic acid *in situ* hybridization (FISH) assays to detect *A. invadans*. Skin ulcers of Atlantic menhaden from populations found in the Pamlico and Neuse River estuaries in North Carolina (USA) were surveyed. Results indicated that affected menhaden were positive for *A. invadans*. Neither the FISH assay nor the PCR assay cross-reacted with other closely related oomycetes. These results provided evidence that *A. invadans* was the primary oomycete pathogen in ulcerative mycosis and demonstrated the usefulness of the assays.

Genome Structure and Transcription

At present, oomycete genome and cDNA sequencing projects have focused on plant pathogenic species from the order Peronosporales (*Phytophthora* and *Hyaloperonospora*) and have yielded tremendous new insights into the pathology of these species. Up to now, no Saprolegniales genome or any other animal pathogenic oomycete has been sequenced. However, a full genome sequence will shortly become available for *S. parasitica* as funding has been secured to sequence an isolate at the Broad Institute in Boston (USA). Comparing the genetic repertoire of *Saprolegnia* with those of the plant pathogenic oomycetes that are already available will provide important insights into the differences and similarities between plant and animal oomycete pathogens.

Preliminary analyses have estimated the total genome size of *S. parasitica* to be around 45 Mb (P. van West, unpublished data). This is about half to a quarter of the size of the plant pathogenic oomycetes that have been sequenced to date (*P. sojae* 95 Mb, *P. ramorum* 65 Mb, *Hyaloperonospora parasitica* 75 Mb and *Phytophthora infestans* 237 Mb) and a similar size to what has been predicted for *S. monoica* (51 Mb) (Mort-Bontemps and Fevre, 1995; Tyler *et al.*, 2006). Mort-Bontemps and Fevre (1995) identified eight chromosomal bands in *S. monoica*, correlating to 16 chromosomes, which were significantly less than

were found in its close relative *S. ferax*, which is thought to contain 21 chromosomes (Mort-Bontemps and Fevre, 1995).

Grayburn *et al.* (2004) have sequenced the mitochondrial genome of *S. ferax*. They found it to be a circular genome of 46,930 base pairs containing a very large inverted repeat of 8618 base pairs. Eighteen open reading frames were found to encode subunits of respiratory complexes I, III, IV and V; 16 encode polypeptides of small and large mitochondrial ribosome subunits; and one encodes a subunit of the sec-independent protein translocation pathway. Furthermore, the protein coding regions are arranged in operon-like clusters including three abutting and two overlapping pairs of reading frames. Interestingly, there were no tRNAs found that allowed translation of any threonine or the arginine CGR codons.

At present, about 1500 expressed sequence tags (ESTs) from *in vitro* grown mycelia of *S. parasitica* have been sequenced and are deposited in a public database (www.oomycete.org) (Torto-Alalibo *et al.*, 2005). Several of these cDNAs were predicted to encode secreted proteins such as those with similarity to fungal type I cellulose binding domain proteins, PAN/apple module proteins, glycosyl hydrolases, proteases, as well as serine and cysteine protease inhibitors, that could function in virulence (Torto-Alalibo *et al.*, 2005). Currently, the van West laboratory is annotating a zoospore/cyst/germinating cyst cDNA library and two interactive cDNA libraries: one cDNA library consists of an RTG-2 trout gonad cell line that has been harvested 8 h post-infection with *S. parasitica*. The second cDNA library originated from a colony of *S. parasitica* that was isolated from skin tissue of an infected Atlantic salmon. Preliminary analysis of the zoospore/cyst/germinating cyst cDNA library has highlighted a few genes coding for putative effector proteins containing an RxLR-motif (van West, 2006). This amino acid motif has been implicated in translocation of effector proteins from several plant pathogenic oomycetes into their host cells (Birch *et al.*, 2006; Whisson *et al.*, 2007). Whether RxLR containing proteins from *Saprolegnia* are also translocated into fish host cells and, if they are, whether they would execute a similar role remain to be investigated.

Pathogenesis and Immunity

Very little distinction has been made between opportunistic and necrotrophic colonization by oomycetes of moribund or dead eggs (Fig 18.1) or wounds and a genuine infection of intact (although undoubtedly stressed) animals and healthy eggs. It is likely that many water moulds that have been described as pathogens are little more than opportunist saprotrophs. Only on species such as *S. parasitica* (Pottinger and Pickering, 1985; Fregeneda-Grandes *et al.*, 2001) and *A. invadans/piscida* (Hatai *et al.*, 1984; Lilley and Roberts, 1997) has pathogenicity been demonstrated experimentally, although often only if hosts are hormonally or artificially stressed.

We do not have a clear understanding of the basis of pathogenicity for S. *parasitica*. It is tempting to suggest that the long hairs associated with the species facilitate infection by enabling the spores to attach to fish more efficiently, although as indicated earlier, it is often those isolates with shorter hairs that appear to be the more aggressive (Fregeneda-Grandes *et al.*, 2001). There have been a number of alternative suggestions for how the long spines might improve pathogenicity and these include reducing rates of sedimentation and allowing the spores to remain at the water–air interface (Hallett and Dick, 1986; Willoughby and Roberts, 1992a), thereby increasing the probability of them encountering a fish host. These observations have promoted the suggestion that the length of hooked hairs is related to pathogenicity (Beakes, 1983; Hatai and Hoshiai, 1993). Indeed, this was a conclusion reached by Manton *et al.* (1951) and Meier and Webster (1954), who considered that the long bifurcate hooks of the secondary cyst of *Saprolegnia* played a role in attachment to the fish host. Roberts (1989) carried out experimental studies to try and prove that these ornamentations were

important in the pathogenicity. They set up a series of challenge experiments using a number of salmonid hosts and showed that *S. parasitica* remained localized on the fish surface for longer periods than the saprophytic *S. diclina*. However, the number and size of hooks are not necessarily good indicators of aggressiveness, with the most highly pathogenic isolates typically possessing relatively small bundles of relatively short stiff hairs (Hatai *et al.*, 1990; Fregeneda-Grandes *et al.*, 2000). Rand and Munden (1993a) found no evidence that attachment of *Saprolegnia* cysts to brook trout eggs was mediated by the cyst-wall appendages and they proposed that attachment was facilitated by the release of an adhesive substance. It seems likely that while long hairs may facilitate uptake and retention to the fish surface, other properties may also be required for a pathogen to breach the defences of a living fish successfully.

Pathology

Early circular or crescent-shaped skin lesions associated with *Saprolegnia* infection in salmonids are often characterized by growths of thin, white or grey threads (Willoughby, 1989). Microscopic examination of hyphal growth reveals the characteristic branched, coenocytic mycelium, with many zoosporangia. The histopathology associated with early, superficial infection in salmonids shows rapid degenerative changes in the epidermis and dermis. More aggressive lesions, with deeper myofibrillar and focal cellular necrosis, spongiosis or intracellular oedema and ultimate sloughing of the epidermis, may follow (Neish, 1977; Pickering and Richards, 1980). Associated inflammatory reactions are absent (Hatai and Hoshiai, 1992). As the oomycetes radiate from the focus of infection, more of the epidermis is destroyed and, consequently, hyphae can penetrate the basement membrane, with growth sometimes continuing into the hypodermis and musculature (Neish, 1977). Thrombi frequently are observed in the blood vessels as a result of the penetrating hyphae. Primary infectious lesions with many hyphae have been reported in the pyloric region of the stomach of amago salmon, and these hyphae may also invade other abdominal tissues (Miyazaki *et al.*, 1977). Furthermore, the gill lamellae and pharynx have been the sites for primary infection of farmed Atlantic salmon fry (Bruno and Stamps, 1987).

Saprolegniasis has been correlated with extensive haematopoietic pathology in brown trout (Álvarez *et al.*, 1988). In addition to a marked lymphocytopenia (Pickering and Pottinger, 1988), significant impairment of the haematopoietic organs has been reported. Lymphoid cell degeneration, cell depletion, vascular alterations within blood vessels and enlargement and hypertrophy of sinusoidal endothelial cells have also occurred (Álvarez *et al.*, 1988). Álvarez *et al.* (1995) recorded histological changes in the thymus and, although the hyphae had not invaded this organ directly, the tissue was oedematous, with epithelial hypertrophy, increased pyknosis and phagocytic activity, involving macrophages and epithelial cells. Considerable changes occurred in the structure of the parenchyma, with large areas showing a marked decrease in cellular density. Most thymocytes were pyknotic, and both epithelial cells and macrophages contained engulfed dead cells. Darkly staining epithelial cells showed cytoplasmic vesicles and clear signs of degeneration, including a vacuolar cytoplasm, with no inflammatory response to invasion.

Copland and Willoughby (1982) reported a loss of integrity of the integument, an oedema of the hypodermis, with degenerative changes in the muscle mass, accompanied by marked myofibrillar degenerative changes in farmed elvers infected with *Saprolegnia*. Severe infection causes swelling in the intermyotomal connective tissue, which has a fenestrated appearance, due to loss of nuclei. In farmed pike, muscle lesions attributed to oomycete infection are uncommon, but hyphae occasionally invade deeper areas, including nervous tissue (Bootsma, 1973). The location of the hyphae suggests that pathogenic *Saprolegnia* generally are not tissue specific.

Saprolegnia lesions in channel catfish initially occur at the site of injury, containing a central zone of either necrotic skin, with fungal mycelia throughout the lesion, or, in more severe lesions, a necrotic core of sloughed tissue, which leaves a crater-shaped cavity (Xu and Rogers, 1991). In some lesions, the epidermis was sloughed completely, leaving the dermis exposed (Xu and Rogers, 1991; Bly *et al.*, 1992). Adjacent tissue becomes infected following the spread of hyphae on the skin surface, and mucus cells present in normal skin are absent in the infected skin.

Oomycetes other than *Saprolegnia* reported as pathogens of wild and farmed fish species include *Aphanomyces* and *Achlya* (Miyazaki and Egusa, 1973; Fraser *et al.*, 1992; Kitancharoen *et al.*, 1995). The early signs of EUS infection caused by *A. invadans* include loss of appetite and fish become darker. Infected fish may float below the surface of the water and become hyperactive, with a very erratic pattern of movement. Red spots may be observed on the body surface, head, operculum or caudal peduncle. As infection develops, large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Most species, other than striped snakehead and mullet, die at this stage. In highly susceptible species, such as snakehead, the lesions are more extensive and can lead to deep penetrating, chronic lesions that penetrate the skin and erode underlying muscle with complete erosion of the posterior part of the body, or to necrosis of both soft and hard tissues of the cranium, so that the brain is exposed in the living animal.

Hatai *et al.* (1994) and Wada *et al.* (1994) observed multiple granulomas within the internal organs and musculature of the dwarf gourami (*Colisa lalia*) infected with *Aphanomyces*. These consisted of mononuclear cells, neutrophils, macrophages and fibrillar structures and were considered to resemble the mycotic granulomatosis recorded in farmed ayu infected with *Saprolegnia* (Hatai, 1980). Multinuclear giant cells were absent in the *Aphanomyces* and *Achlya* infections.

In the eastern USA, Noga *et al.* (1988) assigned an ulcerative mycosis in Atlantic menhaden to an oomycete infection. Advanced lesions consisted of open ulcers containing many hyphae, interspersed with necrotic muscle. Hyphae were surrounded by intense inflammation, with many basophilic granulomas possessing necrotic centres. Some lesions were haemorrhaged and infiltrated by lymphocytes and granular cells. Peduzzi and Bizzozero (1977) speculated that the secretion of lytic enzymes ahead of the hyphae might account for tissue damage. Deeply basophilic granulomas and other haemorrhagic lesions, infiltrated with lymphocytes and occasional multinucleated giant cells, were also observed.

However, it is considered unusual for granuloma formation to occur in association with oomycete infection. Hatchery mortality among juvenile ayu has been attributed to *S. diclina* type 1 infection and diagnosed as a mycotic granulomatosis (Hatai, 1980; Wada *et al.*, 1993). Histologically, numerous hyphae observed in the stomach penetrate into the pyloric caeca and other visceral organs, resulting in severe necrosis. Multinuclear giant cells are abundant in these lesions. Wada *et al.* (1993) recognized that primary infectious lesions in farmed ayu were established initially in the pyloric region, with hyphae invading from the mucous membrane into these regions. Haematoxylin and eosin, periodic acid-Schiff (PAS) and general fungal stains (e.g. Grocott's) are used to demonstrate typical granulomas and invasive hyphae. Early EUS lesions show an erythematous dermatitis with no obvious oomycete involvement. *A. invadans* hyphae are observed growing in skeletal muscle as the lesion progresses, forming a mild chronic active dermatitis to a severe locally extensive necrotizing granulomatous dermatitis with severe floccular degeneration of the muscle. This species elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae. Deeply penetrating ulcers, as well as raised lesions, are characterized by deeply penetrating hyphae surrounded by chronic, granulomatous inflammation (Blazer *et al.*, 1999).

Electron-microscopy studies of channel catfish infected experimentally with *Saprolegnia* revealed the appearance of lesions typically between 7 and 9 days post-infection (Xu and Rogers, 1991). Hyphae penetrated the epidermis and dermis, with necrosis and sloughing in areas next to the hyphae. Damage to fibroblasts and change in the collagen orientation were noted in both naturally and experimentally infected fish. Some epithelial cells were more electron-dense than normal cells and the nuclear membrane in other cells had ruptured, showing an increase in cytoplasmic vacuoles. Earlier, Álvarez *et al.* (1988) had hypothesized that cytoplasmic vesicles observed in the endothelial cells were derived from the oomycete. Xu and Rogers (1991) also noted the destruction of collagen, with significant debris containing melanosomes between the lamellae.

The invasion of brook charr eggs by *Saprolegnia* hyphae was shown, using scanning electron microscopy, to occur from a combination of mechanical pressure and extracellular enzyme digestion (Rand and Munden, 1992). Brook trout eggs exposed to zoosporulating hyphae were colonized by encysted spores, spore germlings and young thalli within 15 min post-exposure (Rand and Munden, 1993a). Some thalli penetrated the outer layer of the chorion and appeared to spread in a lateral direction just beneath the membrane surface. Between 1 and 24 h post-exposure, infected eggs were covered by a few branching hyphae, either spreading over or penetrating the chorionic membrane. However, by 24 h post-infection, a light to moderately heavy mycelial mat covered the egg surface.

Osmoregulatory failure

The primary sequel of uncomplicated saprolegniasis is an osmotic imbalance, due to loss of epithelial integrity and tissue destruction, caused by penetration of the hyphae (Copland and Willoughby, 1982; Noga, 1993b). It is generally accepted that death is due to severe haemodilution, caused by haemorrhage, and the progressive destruction of the epidermis by hyphae (Hatai and Hoshiai, 1994). A significant decrease in serum ions in *Saprolegnia* infected mature brown trout has been correlated with infection, serum osmotic pressure and sodium ion concentration (Richards and Pickering, 1979; Duran *et al.*, 1987). The pathology observed can be attributed directly to the tissue destroyed in the immediate area of the hyphae (Neish, 1977), and the oedema in infected fish can be assigned to osmoregulatory changes (Richards and Pickering, 1979; Noga, 1993a). However, Noga *et al.* (1988) considered that these observations did not explain fully the lesions in deep muscle fibres in menhaden.

Enzyme activity

Peduzzi and Bizzozero (1977) showed that the thalli of certain fish-pathogenic *Saprolegnia* had chymotrypsin-like activity and postulated that this enzymatic activity contributed to pathogenesis. The work was extended by Rand and Munden (1992), who reported high lipase and alkaline phosphatase activity surrounding the thalli of *S. diclina* infesting the egg membrane of brook charr. These enzymes may alter the integrity of the chorionic membrane by solubilizing structural polymers, thereby easing penetration of the thalli. Enzyme changes noted in *Saprolegnia* infected brown trout include lactate dehydrogenase, glutamate–oxyalate transaminase (GOT), glutamate–pyruvate transferase (GPT), creatine phosphokinase, alkaline phosphatase and acid phosphatase, with the presumptive liver enzymes GOT and GPT being elevated, which suggests hepatocellular damage (Duran *et al.*, 1987). Fish dying from *Saprolegnia* infection suffer a severe haemodilution associated with elevated serum enzymes (Noga, 1993a; Hatai and Hoshiai, 1994) and a hypoproteinaemia in several fish species, including brown trout (Richards and Pickering, 1979), Atlantic salmon (Mulcathy, 1969) and coho salmon (Hatai and Hoshiai, 1994). Severe hypoproteinaemia and a significant reduction in the

albumin:globulin ratio is reflected in the electrophoretogram of the serum proteins from infected fish (Richards and Pickering, 1979).

Immunology

Resistance to infectious diseases may be innate or specifically acquired. There are a few reports on the relationship between genetic variation and disease resistance of farmed salmonids (see Chevassus and Dorson, 1990). Nilsson (1992) reported a positive relationship between mortality of replicated families of Arctic charr with *Saprolegnia* infection. Heritability estimates for mortality showed that tolerance to *Saprolegnia* infection by the charr could be improved by selective breeding of fish.

There is increasing evidence that oomycete infections are associated with immunosuppression (Álvarez *et al.*, 1988; Hayman *et al.*, 1992), with many outbreaks occurring after a sharp decrease in water temperature, to levels near the physiological minimum for a particular fish species. Oomycete growth occurs rapidly, with the formation of characteristic skin lesions, accompanied by significant fish mortalities (Bly *et al.*, 1994; Quiniou *et al.*, 1998) and, at temperatures above 12°C, the culture of channel catfish is under threat from outbreaks of *Saprolegnia* spp. Many farmed catfish in southern USA die following such infections, known locally as 'winter kill' or 'winter saprolegniasis' (Bly *et al.*, 1992, 1993, 1994). Affected fish show *Saprolegnia*-associated skin lesions and mucus-depleted skin. Experimental studies demonstrated that this condition involved complex reactions between a rapid decrease in water temperature, *in vivo* immunosuppression, lack of an inflammatory response and the *Saprolegnia* (Xu and Rogers, 1991; Bly *et al.*, 1992; Álvarez *et al.*, 1995). Quiniou *et al.* (1998) noted there was a marked decline in mucous cells in catfish subjected to a water temperature drop of 12°C and there was no recovery of these cells when they were also challenged with *Saprolegnia*. Low temperature-induced mucous loss may explain how cysts attach and infect catfish skin and result in disease. Hayman *et al.* (1992) found that channel catfish were also complement-deficient in the winter months. Serum CH50 values were severely depressed, suggesting that immune and non-immune functions generally associated with complement were not functioning correctly. These authors proposed that the lack of leucocytic infiltration in *Saprolegnia* infected tissue was related, in part, to the inactivity of the complement system, but it was possible that low water temperature also affected the synthesis of complement proteins. Bly and Clem (1992) considered that low water temperature, elevated corticosteroid and an increase in cytotoxic factors secreted by the oomycete might account for immunosuppression. Similarly, Fregeneda-Grandes *et al.* (2009) reported the low prevalence of antibodies observed in *Saprolegnia* infected brown trout that suggested possible immune suppression and the lack of an effective specific immune response against infection.

More recent reports indicate that *Saprolegnia* infections do stimulate an acute phase response in infected fish (Bly *et al.*, 1994; Roberge *et al.*, 2007). Bly *et al.* (1994) suggested that catfish and possibly other fish could clear oomycete infection via a cell-mediated response. When catfish were held at 22°C and injected intramuscularly with viable *Saprolegnia*, the hyphae were destroyed rapidly in a classical foreign-body response (Bly *et al.*, 1994). Álvarez *et al.* (1995) further postulated that the disappearance of the thymic parenchyma in infected brown trout and a combination of some or all of the above factors were related to *Saprolegnia* infection. Roberge *et al.* (2007) identified several Atlantic salmon genes to be upregulated after *Saprolegnia* infection. Among these upregulated genes, several acute phase genes were identified, including genes from the complement pathways, genes encoding proteases which have putative collagenolytic activity that may aid leucocyte transmigration, a gene encoding a G-protein-coupled receptor involved in skin inflammation and TAP2, an ATP-binding cassette transporter that played important

roles in MHC class I antigen presentation. Furthermore, other genes encoding acute phase proteins were upregulated. These included leucocyte chemotoxins, cytokine receptors and agglutination and aggregation factors (Roberge *et al.*, 2007). Further evidence of an active immune response was found by Kales *et al.* (2007), who performed detailed expression analysis with a rainbow trout macrophage cell line that was infected with both live and heat-killed spores and mycelia from *Achlya* and *Saprolegnia*. These authors found that calreticulin and interleukin-8 were moderately upregulated in the challenged macrophage cell line, which suggested that calcium modulating and chemotactic responses had taken place. Furthermore, cyclooxygenase (COX-2) and the cytokines IL-1β and TNFα were strongly upregulated in the presence of *Achlya*, whereas gene expression of some of the class II major histocompatibility (MHII) receptor and associated molecules seemed to be downregulated. This is a very interesting observation, as their experiments were not performed at colder conditions, which would induce a reduced immune response. Consequently, the authors postulate that the oomycete must have, somehow, interfered with the immune response in the cell line.

In summary, despite contradictory reports, there is accumulating evidence that expression of genes encoding acute phase proteins is triggered by oomycete infection in fish, and thus the lack of an immune response seen in other studies may be attributed to a drop in water temperature. However, at this stage, we cannot rule out that oomycetes may also facilitate immune evasion by an as yet unknown mechanism.

Control, Treatment, Prevention and Epizootiology

Oomycetes are transmitted in several ways to fish, including wild and farmed fish, their eggs, the water supply, transport vehicles, movement of staff between aquaculture facilities and farm equipment, such as nets. Transmission occurs directly between fish and/or eggs (Singhal *et al.*, 1987). Several key factors are known to affect both the sensitivity of the fish to infection and the growth of the oomycete. These include pollution, low water levels, overcrowding, mechanical trauma, including handling, the failure to remove moribund and dead fish or ova, changes in hormonal status and the result of infection by other organisms (Piper *et al.*, 1982; Plumb, 1984). In addition, there may also be some seasonal variations in inoculum potential (Hunter, 1975). Among wild fish, redd digging and spawning contribute to physical damage and therefore the possibility of increased infection (Richards and Pickering, 1978). There are at least three lines of defence against *Saprolegnia* infection following the challenge of fish with zoospores (Wood *et al.*, 1988). The skin is the first point of contact for the infective organism, and increased secretion of mucus following contact with secondary cysts may serve to reduce the number of propagules on the fish surface. Pickering (1994) concluded that there was circumstantial evidence linking a decrease in mucification from fish with increased susceptibility to oomycete infection. Willoughby (1989) also suggested this process represented an important defence against infection. Secondly, a morphogen from the external mucus could inhibit mycelia growing from spores (Wood *et al.*, 1988) and, finally, a cellular response was detected in the external mucus. Thus, the mucosal layer acts primarily as a physical barrier to colonization by oomycetes or other infectious agents. Components in the cyst-coat matrix may aid physical entanglement with the fish surface (Beakes *et al.*, 1994a). Willoughby and Pickering (1977) noted that the number of spores on the skin of brown trout was reduced greatly during the first 24 h following exposure of the fish to *Saprolegnia*. Mucus removed from the surface of fish triggered encystment of zoospores from pathogenic strains of *Saprolegnia*, with mycelial growth following rapidly (Pickering and Willoughby, 1982b). As a result of damage to the epithelium and the resulting loss of goblet cells, mucus production ceases and it is at this stage that the

cysts are more likely to attach (Pickering, 1994).

Adhesion of *Saprolegnia* cysts to substrates is believed to involve the production of a non-fibrous conconavalin A-binding material by the oomycete (Beakes *et al.*, 1994a). Discrete points of attachment to the egg are formed and appear raised, which suggests that the associated hyphae are attached firmly to the surface (Rand and Munden, 1993a). Shortly after exposure, encysted spores and young thalli are found on eggs. The development of hyphae, their spread and penetration into the egg are considered important factors for the establishment of infection. Within 1–24 h post-exposure, the egg surface is covered by branching hyphae, spreading over, or sometimes invading, the chorionic membrane. Dead eggs are particularly susceptible and infection may spread rapidly to viable eggs (Rand and Munden, 1993a). Consequently, it is not surprising that chorionic membrane extracts have been identified as factors stimulating positive chemotaxis of zoospores towards live eggs (Rand and Munden, 1993b). A positive chemotactic response towards arginine and alanine was also detected, but no such response was observed to other amino acids or sugars tested. These results indicate that chemotaxis may have an important role in attracting zoospores of *Saprolegnia* towards live salmonid eggs. Rand and Munden (1993b) concluded that chemoattractants might be useful in the development of strategies to control or eliminate *Saprolegnia* among salmonid eggs raised in hatcheries.

Infectious disease outbreaks always pose a risk to farmed stock, despite the quality of husbandry practices employed. The main factor determining whether *Saprolegnia* infection occurs is considered to be the physiological state of the fish (Pickering and Christie, 1980; Cross and Willoughby, 1989). Sexually mature, stressed or damaged fish are the most susceptible to infection. Oomycete outbreaks in hatcheries can result in substantial egg mortality (Smith *et al.*, 1985), as hyphal growth on the chorionic membrane spreads rapidly, especially if dead eggs are not removed. Losses of 10% or more are quite commonplace (Marine Harvest Ltd and Landcatch Ltd, personal communications).

Trade in aquatic animals, especially the movement of fish to countries where they are not indigenous, represents a risk to fisheries and aquaculture (Lilley *et al.*, 1997b). The use of molecular tools (e.g. RAPD-PCR) enabled Lilley *et al.* (1997b) to show that isolates of *A. invadans*, obtained from fish reared in several different countries, had identical nucleotide sequences and could not be differentiated by restriction fragment length polymorphism. Later, Lilley *et al.* (2003) reported that restriction fragment length polymorphisms and sequences of ribosomal DNA confirmed that *A. invadans* was distinct from the other species. This group reported intraspecific relationships examined by random amplification of polymorphic DNA using 20 isolates of *A. invadans* from six countries with a high degree of genetic homogeneity using 14 random ten-mer primers. This provided evidence that the pathogen had spread across Asia in one relatively rapid episode, which was consistent with reports of outbreaks of EUS. Physiological distinctions between *A. invadans* and other *Aphanomyces* species based on a data set of 16 growth parameters showed remarkable taxonomic similarity with the molecular phylogeny.

Treatment and protection

Oomycete overgrowth on developing salmonid eggs (Fig 18.1) and fry is a widespread problem. Overcrowding, handling, temperature changes, parasitism, increased organic loading and sexual maturation increase the likelihood of *Saprolegnia* and other diseases (Toor *et al.*, 1983; Pickering, 1994). Where infections are slight, lesions may be treated with some success. Fish may also recover if they migrate naturally or are moved to estuarine or seawater (Pickering and Willoughby, 1982b; Noga *et al.*, 1988). However, salmonids with significant saprolegniasis normally do not recover once the oomycete penetrates its host, as it

is largely protected from chemical treatments.

Many potential control methods for the oomycetes are reported, but direct comparison between such reports is difficult. The range of factors influencing exposure of fish and particular chemicals has not been standardized. This is particularly relevant where the relationship between an increase in water temperature and the toxicity of malachite green has been examined (Alderman and Polglase, 1984; Alderman, 1985).

The efficacy of anti-oomycete compounds in the presence of excretory products was noted by Willoughby and Roberts (1992a). They found that *S. parasitica* zoospores in pure water were sensitive to a combination of penicillin and streptomycin, but to a lesser extent in waters containing high levels of nutrients resulting from fish excretory products. Interestingly, 0.1 mg/l malachite green oxalate was effective at inhibiting germination of zoospore cysts in pure and nutrient-enriched water, but not in the presence of dense plankton concentrations.

EUS occurs widely in natural waters and ponds and currently there is no fully effective method of control. However, it is possible to prevent and control in ponds by closing the inlet to exclude infected fish, reduce feeding during an outbreak, adding lime and salt to ponds at 400–600 kg/ha, aeration of the water and exchanging water after the situation has improved. In addition, the removal of infected fish, drying of ponds before restocking and disinfection of contaminated equipment should be standard practice. Furthermore, hypochlorite can be used at 50 mg/l to disinfect the pond for 7–10 days before stocking.

Chemical treatment

The development of a reliable method to infect fish experimentally is essential in order to determine the efficacy of various treatments. The search for alternative methods and compounds for the control of oomycete outbreaks has increased, and the efficacies of many potential oomyceticides have been tested using *in vitro* screening methods. Bailey (1983a,b) outlined a method for screening aquatic fungicides using agar plugs containing hyphae removed from the edge of actively growing colonies. Plugs were placed into depressions containing the test compound. This approach enables many compounds to be screened in a reasonable time and helps identify those compounds with oomyceticidal activity. Howe *et al.* (1998) reported on a method to induce saprolegniasis systematically in channel catfish using a combination of low-temperature and abrasion stress. Furthermore, it was found that *in vitro* testing correlated well with *in vivo* testing of surface infections of fish. Bailey (1983a) recommended that the mean activity of each test should be carried out in triplicate for each compound and be compared with the activity obtained for malachite green. Theoretically, selected oomyceticides should control oomycete growth for at least 48 h (Bailey, 1983a). For *in vitro* testing, he advocated using cornmeal agar as a suitable medium for testing potential oomyceticides, as it was readily available, maintained a stable pH and promoted growth. In preliminary screening of *Achlya* and *Saprolegnia*, a culture temperature of 20±2°C, with a pH of 6±0.2, was proposed. Other recommendations for standardization were also outlined, and the use of this method or with slight variations has been adopted. The acceptance of compounds with oomyceticidal potential depends on the adoption of these specific criteria.

Bailey and Jeffrey (1989) examined more than 200 compounds to determine their inhibitory activity against potentially pathogenic oomycetes, such as *A. flagellata* and *S. hypogyna*. They established minimum inhibitory concentrations for each compound they examined. Oomycete growth (colony diameter) was categorized into three levels of activity, high (<10 mg/l), moderate (>10–100 mg/l) or low (>100 mg/l). More than 50% of compounds tested were unsuitable as oomyceticides. After further testing, the activities of the remaining compounds were tested against oomycete infected rainbow trout eggs.

Further work, including the testing of candidate compounds, undoubtedly is required, as new regulations are introduced that limit the compounds used in aquaculture. This is particularly relevant in the European Union (EU), where all compounds used as medicines must be given official approval through a licence or registration before their use is permitted. Since the selection of aquatic oomyceticides frequently is based on comparative studies using malachite green as a reference compound (Bailey and Jeffrey, 1989), it is appropriate that a brief review on the status of this compound is included.

Malachite green

Malachite green was used successfully for treating all infectious stages of *Saprolegnia* in fish aquaculture facilities worldwide (Foster and Woodbury, 1936; Olah and Farkas, 1978; Bailey, 1984; Alderman, 1985; Willoughby and Roberts, 1992a). However, since the turn of the century, malachite green has been banned worldwide due to its carcinogenic properties. Several studies associated with malachite green identified toxicological (Bills *et al.*, 1977; Meyer and Jorgenson, 1983) and potentially mutagenic properties (Clemmensen *et al.*, 1984; Fernandes *et al.*, 1991; Srivastava *et al.*, 2004). In addition, there were implications for this compound as a teratogen (Steffens *et al.*, 1961; Meyer and Jorgenson, 1983) and a tumour promoter in animals (Fernandes *et al.*, 1991). Furthermore, malachite green was reported as irritating to mucous membranes and mutagenic in the *Salmonella* microsome mutagenicity test (Clemmensen *et al.*, 1984). Fish subjected to excessive exposure to malachite green may also show respiratory distress (Alderman, 1994). Meyer and Jorgenson (1983) reported spinal, head, fin and tail abnormalities in trout fry hatched from eggs exposed to standard repetitive malachite green treatments, although the dose was higher than that used in aquaculture.

The chemical controlled *Saprolegnia* infections on eggs with minimal mortality if used at concentrations between 3 and 5 mg/l for a 60 min exposure (Bailey, 1983a; Marking *et al.*, 1994a,b). During continuous exposure, malachite green was effective at killing zoospores and inhibiting hyphal growth at 0.25 mg/l (Willoughby and Roberts, 1992a). Furthermore, the inhibition of zoospore germination (0.06–0.50 mg/ml) and hyphal growth (0.5–2.0 mg/ml) was reported for *Aphanomyces*, *Achlya* and *Saprolegnia* (Srivastava and Srivastava, 1978; Hatai *et al.*, 1994; Yuasa and Hatai, 1995b). Other studies reported similar values (Cline and Post, 1972; Srivastava and Srivastava, 1978; Hatai *et al.*, 1994; Yuasa and Hatai, 1995b). Willoughby and Roberts (1992a) considered that a contact time of 15 min at 10°C would be sufficient to kill mycelia established on fish within the previous 24 h, and therefore control could be achieved through a single flush treatment of malachite green.

Formalin

Formalin, an aqueous solution containing approximately 37% formaldehyde by weight, has been widely used to control oomycete infections in aquaculture (Cline and Post, 1972; Walser and Phelps, 1993). Effective control of oomycete outbreaks has been recorded for rainbow trout following a 60 min exposure at 250 mg/l (Bailey and Jeffrey, 1989). Similar concentrations of 150–300 mg/l gave effective control of *Saprolegnia* in rainbow and brown trout (Cline and Post, 1972). Marking *et al.* (1994a) showed that 250 mg/l formalin prevented oomycete infections on eggs. At 1000 mg/l, a decrease in existing infection and an increase in hatching rates were also recorded. Schreier *et al.* (1996) concluded that *S. parasitica* was controlled on rainbow trout eggs that received prophylactic formalin treatments on alternate days. Work by Barnes *et al.* (2000, 2002) suggests that to achieve a substantial decrease in the hand-picking of dead eggs, daily formalin treatments of 1667 mg/l for 15 min are required.

In laboratory challenge tests, Bly *et al.* (1996) found that formalin could be used

prophylactically to control winter saprolegniasis in channel catfish, inhibiting *Saprolegnia* zoospore production at 12.5 mg/l and being suppressive at 7.5 mg/l. Walser and Phelps (1993) reported an improvement in the hatching of catfish eggs relative to untreated eggs, following a twice-daily flush treatment of formalin at 400 mg/l. However, other studies showed that lower concentrations of around 250 mg/l were also effective as flush treatments for this species (Rogers, 1985; Meyer and Schnick, 1989). Currently, formalin is the only registered oomyceticide available for use in fish culture in the USA, but it is limited to use on eggs of salmon, trout and escoids (Marking *et al.*, 1994a). Despite efforts in the USA, through the Aquatic Animal Drug Approval Partnership Programme, a permit to extend the registration to include other species of fish and eggs, concern regarding its safety and the effect of the effluent on the environment remain (Meyer and Schnick, 1989). Such concerns make it unlikely that formalin will be considered for widespread use in the short term and, in fact, it is likely to be banned in the future.

Hydrogen peroxide

Hydrogen peroxide is effective against a variety of organisms, such as bacteria, yeasts, viruses and fungal and oomycete spores, and is potentially an important oomyceticide for fish culture, exhibiting little environmental impact (Schreier *et al.*, 1996). This compound has also been used as a bath treatment for sea lice (Johnson *et al.*, 1993; Bruno and Raynard, 1994). Several groups, including Dawson *et al.* (1994), Marking *et al.* (1994a), Waterstrat and Marking (1995) and Schreier *et al.* (1996), have reported on the effectiveness of hydrogen peroxide for controlling *Saprolegnia* on developing eggs of rainbow trout and Chinook salmon (*O. tshawytscha*). Preliminary tests by Dawson *et al.* (1994) noted that exposure of eggs to a prophylactic treatment of 250–500 ml/l hydrogen peroxide (based on 100% active ingredient) for 15 min, on alternate days, inhibited infections on healthy eggs. However, treatments of 1000 mg/l were necessary to control infection where 10% of the eggs were infected. Dawson *et al.* (1994), Marking *et al.* (1994a), Schreier *et al.* (1996) and Barnes and Gaikowski (2004) reported an increase in the hatch rate of eggs that had received a treatment of hydrogen peroxide at a concentration of 1000 mg/l.

Rach *et al.* (1988) reported the mean percentage egg hatch for warmwater and coldwater fish species was higher with 1000 µl/l hydrogen peroxide than in untreated controls. Howe *et al.* (1999) tested the efficacy of hydrogen peroxide for preventing or controlling mortality associated with saprolegniasis in channel catfish. Saprolegniasis was induced systematically in channel catfish so various therapies could be evaluated in a controlled laboratory environment. Both prophylactic and therapeutic hydrogen peroxide treatments of 50, 100 and 150 ml/l/1 h were administered every other day for seven total treatments.

Gaikowski *et al.* (2003) examined the efficacy of hydrogen peroxide in controlling saprolegniasis on eggs of walleye (*Sitzostedion vitreum*), white sucker (*Catostomus commersoni*) and paddlefish (*Polydon spathula*). Hydrogen peroxide treatments of 500 mg/l either increased egg hatch or were as effective as physical removal of infested eggs in controlling mortality. Although treatment with formalin at 1667 mg/l increased significantly the per cent eye-up of walleye eggs compared with that of those treated with hydrogen peroxide at 500 mg/l, the difference was only 1.9–2.6%. Overall, treatments of 50 ml were found to be the safest and most effective of those tested. Mortality with this concentration ranged from 16% in therapeutic tests to 41% in prophylactic tests. The statistical model employed estimated that the optimum treatment concentration for preventing or controlling mortality, reducing the incidence of infections, and enhancing the recovery of infected fish was 75 µl H_2O_2/l.

Hydrogen peroxide has also been shown to control oomycete infections on adult Chinook salmon, using a flow-through dose of 25 mg/l. In the USA, the Food and

Drug Administration (FDA) has classified hydrogen peroxide as a low regulatory priority (LRP) for the control of oomycetes on all species and life stages of fish (Schreier *et al.*, 1996). This has enabled its use at concentrations of up to 500 ml/l as an oomyceticide without an INAD permit or a new animal drug application (NADA).

A commercial sanitizing agent containing 20% hydrogen peroxide and 5% peracetic acid has also been effective in decreasing oomycete infection on rainbow trout eggs and subsequent hatch rate, following a treatment at 100 mg/l for 60 min (Marking *et al.*, 1994a). However, toxicity to trout eggs was recorded in this study at a concentration of 250 mg/l.

Sodium/calcium chloride

A mixture of sodium and calcium chloride (26:1), applied at 20 g/l for 1 h on three occasions in 1 week, resulted in reduced egg mortality between 6.2 and 9.7% (Edgell *et al.*, 1993). These results compare favourably with 5.2–8.8% for 1 mg/l malachite green tested in an identical manner. Marking *et al.* (1994a) and Schreier *et al.* (1996) showed a decrease in *Saprolegnia* infection of trout eggs and an improved hatching rate using a NaCl bath at 30 g/l. At 20 g/l, the salt mixture was as effective for the control of *Saprolegnia* on uneyed eggs as malachite green. Edgell *et al.* (1993) found an increase in the observed abnormalities among alevins exposed to malachite green (16%) when compared with the salt solution (9.3%). However, they speculated that the increase in coagulated yolk observed within the yolk sac after treatment was unlikely to be serious in terms of alevin development. These authors also suggested that rearing alevins in soft water or with other stress-associated factors might induce or contribute to a higher mortality in the treated group. Oomycete control seems effective with these combined salt solutions, without causing any increase in yolk-sac abnormality. However, at 25 mg/l or above, the salt solutions cause the death of Chinook salmon eggs. Pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) may have a higher salt tolerance. Future research into the use of salt solutions for oomycete control should consider the efficacy, logistics and costs associated with the treatment of large groups of eggs (Edgell *et al.*, 1993).

Seawater flush

A seawater flush into aquaculture facilities offers an inexpensive means of limiting oomycete growth and has been employed successfully (Taylor and Bailey, 1979). A 2–3 h flush of seawater for 5 out of 7 days over 10 weeks controlled *S. diclina* on eggs of pink salmon effectively. In gravel incubators, there was also a higher survival of treated eggs to the fry stage, compared with tray incubators, where no difference in survival levels was recorded. When compared with malachite green, no difference in the survival of eggs was reported over several years of testing. Taylor and Bailey (1979) suggested that, where seawater was available, this method of control could be investigated.

Copper

Compounds containing copper are usually effective as fungistatic agents when used at high concentrations (Somers, 1967; Richmond, 1977). Rainbow trout exposed continuously to sublethal concentrations of copper and challenged with *S. parasitica* spores showed changes in total immunoglobulin levels and the mitogenic responses of pronephric lymphocytes (Carballo *et al.*, 1992). Although this compound induced notable stress, manifested in enhanced plasma cortisol levels, there was no alteration to the mitogenic response of lymphocytes from the pronephros to phytohaemagglutinin (PHA), ConA or lipopolysaccharide (LPS) measured at 1, 3 and 8 h and 1, 3, 7, 14 and 21 days post-exposure. After 21 days, fish were challenged with *S. parasitica* and the same immunological parameters were analysed at 3 h and

7, 14 and 21 days after challenge. No changes in the immune parameters were observed, except an increase in the immunoglobulin levels. Saprolegniasis was not present in fish exposed to copper and a low number of infected fish were observed in the control group. The apparent lack of correlation between stress and the immune response during copper exposure may suggest a direct effect of this toxicant, rather than via a stress-mediated mechanism. Copper is reported as immunotoxic and is considered to act by decreasing both humoral and cellular immune responses, reducing circulating lymphocytes and the phagocytic response. Further work by Carballo *et al.* (1995) into the sublethal effects of copper and other compounds on juvenile rainbow trout and their subsequent challenge with *S. parasitica* was carried out using sublethal concentrations of copper (0.25 mg/l), cyanide (0.07 mg/l), ammonia (0.50 mg/l) and nitrite (0.24 mg/l). After 24 h, no enhancement of infection by *S. parasitica* was recorded. Similar increases in raised cortisol levels were found for the four chemicals tested and were used as an indicator of the stress response. Fish that had cortisol levels <370 ng/ml showed an increase in susceptibility to saprolegniasis, while only 24% of the fish with cortisol levels >370 ng/ml were infected (Carballo *et al.*, 1995).

Iodophores

Buffered bicarbonate iodophores are often used to disinfect eyed ova; they are generally applied within 1 h following fertilization. The aim is to destroy potentially infectious agents, including oomycetes on the egg surface. Eggs are agitated gently in the disinfectant bath for 5–15 min and then rinsed thoroughly. A gentle, even flow of water over the eggs ensures sufficient oxygenation. Alderman and Polglase (1984) confirmed the effectiveness of iodophores commonly used in fish farming (e.g. Wescodyne and Buffodyne) at their recommended concentrations of 100 mg/l available iodine, although in their study exposure was 30 min as opposed to the normal 15 min. The potential of iodine as an oomyceticide for eggs of rainbow trout (Marking *et al.*, 1994a) and channel catfish (Walser and Phelps, 1993) has been examined. Iodine administered twice daily as a flush at concentrations of 50, 100 or 200 mg/l increased the hatching rate of channel catfish eggs. Their potential for further development was considered limited due to the high concentrations required. Although iodophor baths are sometimes reused until the strong yellow colour has faded, the iodophor becomes ineffective at 25 mg/l (Ross and Smith, 1972) and must be changed regularly to maintain efficacy. When treating a large number of fish, a bath or flush method is the most economic, but, for small numbers of fish, a dip treatment of around 30 s is advised. Treatment with buffered iodine following water hardening of eggs is permitted as an LRP by the FDA in the USA and other countries when used at a level of 100 mg/l for 10 min as a disinfectant (Marking *et al.*, 1994b).

Bronopol (Pyceze©)

Bronopol (2-bromo-2-nitro-propane-1,3-diol), formulated as Pyceze is licensed in some countries to treat oomycete infections and reported as effective in protecting predisposed fish from infection by *S. parasitica* when administered as a daily bath/flush treatment at concentrations of 15 mg/l and greater (Pottinger and Day, 1999). Pyceze was also demonstrated to protect fertilized rainbow trout ova from *S. parasitica* challenge when administered as a daily bath/flush treatment at concentrations of between 30 and 100 mg/l. Branson (2002) reported on the efficacy of bronopol against infection of rainbow trout broodstock with *Saprolegnia* using a bath treatment of 20 mg bronopol/l.

Chitosan

Min *et al.* (1994) exposed oospores and vegetative hyphae from *S. parasitica* to chitosan (a deacetylated form of chitin), reporting

complete inhibition by 0.06 and 0.05% chitosan, respectively. Yuasa and Hatai (1995b) also reported on the effectiveness of chitosan for *S. parasitica* and *A. piscida*, finding that it acted as a competitor for calcium-binding sites on the cell surface. Min *et al*. (1994) and Yuasa and Hatai (1995b) considered that these findings warranted further investigation into the application of chitosan for preventing saprolegniasis. The antimycotic activity of modified chitosans indicated that methylpyrrolidinone chitosan, *N*-carboxymethyl chitosan and *N*-phosphonomethyl chitosan exerted effective mycostatic action against *S. parasitica* with no radial growth for 50 h at 20°C (Muzzarelli *et al.*, 2001).

Ozone

Treatment with ozone is effective in reducing saprolegniasis in hatcheries. Forneris *et al*. (2003) reported treatment with ozone increased egg hatching from 42.6 to 49.1% with a dose of ozone from 0.01 to 0.2 mg/l. A dose of 0.3 mg/l applied every second day represented the threshold of toxicity.

The use of rotting barley straw under aerobic conditions in a laboratory bioassay has been reported to stop mycelial growth of some species of *Saprolegnia* (Cooper *et al.*, 1997). At present, this approach has not been examined under hatchery conditions.

Husbandry and good practice

The main methods of oomycete control on eggs involve removal of dead or infected eggs at regular intervals and chemical bath treatments. However, both approaches are generally time-consuming and therefore costly in terms of staff time. Other husbandry practices employed to reduce the likelihood of an outbreak include the use of an elevated water flow to roll fish eggs. Rach *et al*. (1995) held uninfected and *Saprolegnia* infected rainbow trout eggs at various flow rates. Eggs maintained at a flow rate of 300 and 600 ml/min showed no rolling, a higher rate of infection and reduced hatching success. At 1200 ml/min, the eggs were lifted into the water column and rolled moderately and hatching was increased significantly, without oomycete growth. The success of this physical method is dependent on maintaining flow rates at levels that induce a moderate rolling of the eggs. Other areas considered, which achieved variable degrees of success, included the exploitation of biological control, using bacteria, protozoa or crustaceans that feed on or parasitize oomycete hyphae (Oseid, 1977; Willoughby and Roberts, 1992b) and the use of ultraviolet irradiation (Kokhanskaya, 1973; Sako and Sorimachi, 1985). Overall, the most successful strategy for the control of *Saprolegnia* and similar infections in farmed fish is a combination of management techniques and chemical bath treatments. The commercial use of bacterial antagonists seems unlikely, as antibiotics and other chemicals are used in commercial fish farming, and *Pseudomonas* sp. and *P. fluorescens* are recognized causal agents of bacterial haemorrhagic septicaemia in fish (Roberts and Horne, 1978; Nakatsugawa and Iida, 1996).

Non-chemical treatment methods

Ultraviolet irradiation

Ultraviolet irradiation has been used successfully to kill viruses and bacteria in freshwater systems (Sako and Sorimachi, 1985; Liltved and Landfald, 1996), but this treatment seems ineffective towards *Saprolegnia* hyphae *in vitro* (Sako and Sorimachi, 1985). However, the technology of using UV light, ozone and hydrogen peroxide in combination to prevent saprolegniasis on eggs has shown promise in Finnish trials (Rahkonen and Koski, 2002).

Probiotics

Several studies have identified antagonists/probiotics to *S. parasitica*, including a variety of *Pythium*, *Rhizophthora* and *Pseudomonas* species (Hatai and Willoughby, 1988; Petersen *et al.*, 1994; Hussein and Hatai,

2001). Hatai and Willoughby (1988) reported that the bacterium *P. fluorescens* could strongly inhibit the radial growth of *S. parasitica in vitro*, and natural control might therefore be feasible. Oomycete inhibition attributed to this bacterial antagonist/probiotic has been suggested to result from the production of an antibiotic by the bacteria (Gurusiddaiah *et al.*, 1986) or from an efficient iron-capturing siderophore that deprives the oomycete of this essential ion (Weller and Cook, 1983). However, Bly *et al.* (1997) reported that inhibition was not associated with culture supernatants, but might depend on the secretion of a chemical or nutrient acting between the bacteria and the *Saprolegnia*. It is possible that *Saprolegnia* may remove a nutrient or secrete a chemical that the bacteria recognize, and then they react by secreting an inhibitor.

The potential therapeutic application of *Aeromonas* media as a probiotic for the control of winter saprolegniasis in the eel (*A. austalis*) has been evaluated by Lategan and Gibson (2003) and Lategan *et al.* (2004). They reported recovery of fish suffering from saprolegniasis, with temperature appearing to be a factor regulating the effect of these bacteria. The commercial use of bacterial antagonists seems unlikely as antibiotics and other chemicals are used in commercial fish farming, and *Pseudomonas* sp. and *P. fluorescens* are now recognized causal agents of bacterial haemorrhagic septicaemia in fish (Roberts and Horne, 1978; Nakatsugawa and Iida, 1996).

Biological control

A few reports highlight the potential of naturally grazing organisms, such as amphipods, to control oomycete outbreaks. Oseid (1977) noted *Gammarus pseudolimnaeus* and the isopod *Asellus militaris* feeding on *Saprolegnia* growth on dead eggs. In laboratory studies, *G. pseudolimnaeus* prevented oomycete growth on developing eggs from walleye (*Stizostedion vitreum*), at a ratio of one invertebrate to ten fish eggs. Although some live eggs were consumed, this approach demonstrated an improvement over batches where dead eggs were removed by hand. *A. militaris* also controlled growth on eggs, but here no difference was noted between these eggs and the hand-picked controls. Oomycete parasitism was examined by Willoughby and Roberts (1992b), who reported that *Woronia polycystis*, an obligate pathogen of *Saprolegnia*, infected both *S. parasitica* germlings and hyphae *in vitro*. Further development of the potential use of biological controls such as these has not been reported.

Immunization and vaccines

In teleosts, adaptive immune responses are well developed, and specifically acquired antibodies have been demonstrated towards a variety of organisms following artificial immunization (Leong, 1993). However, there are currently no vaccines available for saprolegniasis or other water-mould diseases of fish. Naturally occurring serum proteins have been demonstrated to react with mycelial extracts and culture filtrates in several fish species, and Seelinger (1960) has shown that such reactions between oomycete antigens and antisera are not specific. Any vaccine development for saprolegniasis might best concentrate on stimulating the immune system at the mucosal surface. However, the majority of fish are likely to have been exposed to *Saprolegnia* naturally, due to the prevalent nature of the freshwater water mould (Pickering and Willoughby, 1982b). Beakes *et al.* (1994b) postulated that the specific surface glycoprotein on the cyst-coat spines might represent epitopes to which antibodies might be directed, thereby reducing the 'stickiness' of the spores and their subsequent attachment to the fish surface. However, Burr and Beakes (1994) demonstrated that monoclonal antibodies raised against secondary cyst-coat matrix components of *S. parasitica* were not specific and reacted with all *Saprolegnia* genera plus *A. astaci* and *Achlya* sp., but not members of the Peronosporoicetidae.

Recently, Fregeneda-Grandes *et al.* (2007b) tested whether brown trout (*S. trutta*) showed an adaptive immune response after injection with protein extracts from a pathogenic isolate of *S. parasitica*. They were able

to detect specific antibodies raised against *S. parastica* using a range of techniques. These findings are very promising as they may open possibilities to generate vaccines against *Saprolegnia* and other pathogenic water moulds. At present, fish are vaccinated routinely in the aquaculture industry against a range of bacterial diseases. For example, vaccines against the bacteria *Aeromonas salmonicida*, *Yersinia ruckeri*, *Vibrio anguillarum*, *V. ordalii* and *V. salmonicida* have been available for several years (Gudding *et al.*, 1999). Currently a few laboratories are searching for good antigenic oomycete targets that generate a strong immune response in fish, which eventually could be used to immunize fish.

Conclusions and Recommendations for Future Studies

Our understanding of the taxonomy of the Saprolegniaceae is beginning to be advanced by the application of molecular techniques. Molecular studies comparing the sequences of conserved genes show that the oomycetes have their phylogenetic origins with the chromophyte algae within a broader group that also encompasses dinoflagellates, apicocomplexans and ciliates, rather than with the true fungi (Harper *et al.*, 2005). Molecular techniques are also enhancing our taxonomic knowledge within the oomycetes. Taxonomic analyses using the ITS and/or large ribosomal subunit gene sequences in the Saprolegniaceae (Leclerc *et al.*, 2000; Petersen and Rosendahl, 2000) and RFLP and RAPD analyses in the genus *Aphanomyces* (Lilley *et al.*, 2003) are helping to resolve genus and species level concepts. Many traditional genera, particularly *Achlya*, are not monophyletic assemblages and the genus *Saprolegnia* is also likely to be problematic (Hulvey *et al.*, 2007). However, these preliminary studies have indicated that the two important fish pathogens, *S. parasitica* (Molina *et al.*, 1995; Leclerc *et al.*, 2000) and *A. invadans* (Lilley *et al.*, 2003), both appear to be well-supported taxa, although it now appears that the latter species is synonomous with *A. piscida* (Diéguez-Uribeondo *et al.*, 2009). It is hoped these approaches will soon lead to the development of diagnostic kits specific for *S. parasitica* and subgroups that can be applied in the absence of any reproductive structures. The importance of specific surface topography recognition at the host surface during the initial interaction with the disease agent has been demonstrated. Future studies of such interactions in oomycetes might be directed towards the molecular analysis of cell-surface components, using monoclonal antibodies, and the identification of the cell types involved in host defence. Monoclonal antibodies are likely to provide precise tools for probing and identifying receptor functions of antigens and therefore represent an area that would benefit from further development.

Future availability of the genome sequence for *S. parasitica* and other sequencing programmes should permit significant advances in the understanding of fundamental molecular processes. Furthermore, comparative genomics should shed light on the evolution of pathogenicity in the oomycetes and the wider chromalveolate lineage. For example, do plant and animal pathogens share similar pathogenicity factors and infection strategies? Early indications have revealed surpising similarities in effector protein sequences between *Saprolegnia* and plant pathogenic oomycetes (Phillips *et al.*, 2008).

Many researchers have screened chemicals and advised on their suitability as oomyceticides. Despite this, no agent has been identified which matches the effectiveness of malachite green, although hydrogen peroxide, formalin and NaCl show some potential and prophylactic bath treatments using a buffered iodophor are apparently successful for eggs. Clearly, there is a need for safe and effective oomyceticides for use in aquaculture, and development in this area remains a priority.

Studies identifying mechanisms of immunostimulation in immunosuppressed fish may help to develop a vaccine or therapeutic approach for controlling saprolegniasis. This will require research into the

absence of leucocytic infiltration in infected tissue seen in the winter months, its relationship to the inactivity of the complement system and an understanding of the connection between environmental temperature, water moulds and the nature of the immune response. Studies aimed at producing antibodies to specific surface components, thereby reducing the 'stickiness' of the spores and their attachment to the fish surface, would be beneficial.

There is sufficient evidence to show that the infestation of fish eggs results from a chemotactic response of zoospores to compounds associated with the eggs themselves. Further work is necessary to identify the role of specific compounds, so that it may become practical to manipulate these chemical messengers, allowing the development of more targeted, specific control measures.

Acknowledgement

We thank Dr Andrew Phillips (University of Aberdeen, Scotland) for helping with the preparation of Fig. 18.4.

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19 *Ichthyophonus*

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Introduction

Ichthyophoniasis, due to infection with *Ichthyophonus hoferi* Plehn and Mulsow (1911), has been known in fish since the end of the 19th century (von Hofer, 1893). The disease is of economic significance, in both fish cultivation and wild fisheries, and has a wide host and geographical distribution. It has been the subject of several comprehensive reviews (Reichenbach-Klinke, 1954–1955; Dorier and Degrange, 1960; McVicar, 1982; Rand, 1990, 1992; Sindermann, 1990). Since then, there have been further and particularly significant developments in knowledge of the disease. Major new epizootics have occurred in the North Sea, western Baltic Sea and north-east Pacific and these have stimulated new research into the biology and epizootiology of the disease (Anon., 1991, 1993; Rahimian, 1994; Rahimian and Thulin, 1996; Spanggaard, 1996; Mellergaard and Spanggaard, 1997; Kocan *et al.*, 2003, 2004). During the past 20 years, research has been focused extensively on the Pacific North-west, where the most recent outbreaks causing economic, political and public concern have occurred. Taxonomically, *I. hoferi* was placed initially with the protozoans, then with the fungi and, more recently, within a group intermediate between the two. These most recent developments can be attributed to the application of molecular deoxyribonucleic acid (DNA) technology. Spanggaard *et al.* (1996) and Ragan *et al.* (1996) considered it a member of a lower protistan group. Further clarification has since been obtained of the distinct nature of the genus *Ichthyophonus* within the class Ichthyosporea in the subphylum Choanozoa (Cavalier-Smith, 1998; Ragan *et al.*, 2003), separate from the related but equally distinct genus *Dermocystidium* (Pekkarinen *et al.*, 2003). Less information is currently available on the exact relationships of other organisms closer to *Ichthyophonus* within the Ichthyosporea.

Because of its perceived fungal associations, the terminology normally used to describe the various development stages of *Ichthyophonus* has naturally closely followed that of the mycologist, but some reappraisal would now seem appropriate. Nomenclature is often a controversial area in descriptive biology, often with complex and highly specific language being developed and defended by specialists in their subject areas. However, as most researchers only need to be able to identify the structures being discussed in a report, the use of taxon-specific terminology is avoided in this review. Wherever possible, straightforward descriptive terms are used, although it sometimes proves necessary to

© CAB International 2011. *Fish Diseases and Disorders* Vol. 3:
Viral, Bacterial and Fungal Infections, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

avoid further confusion by cross reference to established terms.

Cause of Ichthyophoniasis

Riddell and Alexander (1911) suggested that *Ichthyophonus* might be a secondary invader, masking a possible primary bacterial agent that was responsible for the debilitating condition in plaice, *Pleuronectes platessa*. Since then, no evidence to support this hypothesis has been provided. It will seldom be possible to determine accurately the role that any given pathogen has in a disease process, as disease-determinant factors are typically multifactorial and usually interact significantly in a complex manner. It is generally evident that fish suffering from any debilitating disease often have an increased susceptibility to other infective agents or for there to be a change in the virulence of previously dormant infections. It therefore is largely academic whether or not an infection such as *I. hoferi* initiates the disease process. In the applied scientific field, it is more important to establish if a particular infection contributes significantly to mortality and so becomes the disease of concern. The widespread occurrence of *I. hoferi* in moribund and dead fish, the ease of establishing experimental infections and of maintaining these sequentially in different species of fish, the re-isolation of infective stages and the development of typical pathology in each pass (e.g. McVicar and McLay, 1985) together suggest a major role of this infective agent in the disease process.

Host Range

The wide range of host species from which *Ichthyophonus* and *Ichthyophonus*-like infections have been reported raises the question whether only one species is involved and whether the identification has been accurate. Susceptible hosts include marine and freshwater crustaceans, elasmobranch and teleost fish, amphibians, reptiles and piscivorous birds (Reichenbach-Klinke, 1954–1955, 1957; McVicar, 1982). Humans or other mammals are apparently not at risk from infection. Spanggaard (1996) and Spanggaard and Huss (1996) reported that infection trials with mice showed no indication of toxicity or pathogenicity and that no cases of human infection had been reported. Experimental support was provided by Spanggaard and Huss (1996), who found survival of the parasite to be less than 3 min at 40°C. Fish predominate as hosts, and Reichenbach-Klinke and Elkan (1965) reported infections in 35 marine fish species and 48 freshwater species. More recently, Spanggaard *et al.* (1994) noted more than 80 fish species were susceptible to infection. The literature therefore indicates low parasite–host specificity in fish (McVicar, 1982). Consequently, new fish host records and a host list are probably of little scientific significance and they may largely reflect whether a particular species of fish has been examined sufficiently and appropriately. It is noteworthy that records from elasmobranchs are rare (Reichenbach-Klinke, 1957).

In view of the strong historical link with teleosts, and with new diagnostic techniques now available, the records of *Ichthyophonus* from other groups of hosts need to be reconsidered carefully. Also, the possibility of contamination, accidental and temporary infections, transport, and paratenic or dead-end hosts cannot be ignored. Most studies have used morphological features for identification as *Ichthyophonus* or *Ichthyophonus*-like. The application of molecular genetics would be appropriate to determine if these organisms are the same as in fish. Many infectious conditions such as mycobacteriosis and non-infectious insults can elicit a granulomatous host reaction similar to that caused by *Ichthyophonus*. The risks are evident from assuming that such types of lesions are indicative of *Ichthyophonus* infection without sufficient evidence that it has been the actual cause of the reaction. It is not possible to determine the extent to which misidentifications have appeared in the literature without a thorough reappraisal of the original material used, or an assessment made of new material from the same host species and geographical locality.

Apstein (1911), Chatton (1920) and Jeeps (1937) described *Ichthyophonus* (or *Ichthyosporidium* as it was then called) in *Calanus* and other copepods but, based on morphological and culture studies, Torgersen *et al.* (2002) considered that parasite unlikely to be *I. hoferi* and more likely to be a specific *Calanus* pathogen. Similarly, the record of *Ichthyophonus* from a fish-eating bird noted by Chauvier and Mortier-Gabet (1984) could benefit from a re-evaluation. There are an increasing number of records of *Ichthyophonus*-like infections from various amphibians and reptiles (Chauvier, 1979; Herman, 1984). Mikaelian *et al.* (2000) reported *Ichthyophonus*-like infections in various wild amphibians in Quebec, Canada, and with four new host records, suggested that ichthyophonosis was enzootic in amphibians in that area. Sherman (2008) and Ware *et al.* (2008) found similar infection in newts (*Notophthalmus viridescens*) in other parts of North America. The latter authors noted large spores, often enclosed by granuloma, which 'resembled those caused by the fish pathogen *Ichthyophonus hoferi*'. Raffel *et al.* (2006) reported several lines of evidence that suggested a role for the amphibian leech (*Placobdella picta*) in *Ichthyophonus* sp. transmission, which contributed to mortality in red-spotted newts (*N. viridescens*) and several frog species in North America.

Geographical Distribution

Ichthyophonus has been recorded from many temperate and some tropical waters throughout the world, both north and south of the equator (McVicar, 1982). Most of the older records from wild fish populations are from the North Atlantic and Japan, particularly from continental and inshore waters (McVicar, 1982), but the disease has been detected in numerous other areas. The deep-sea pelagic fish, *Scopelogadus beanii*, had an *Ichthyophonus*-like infection throughout the species' geographic range in the Atlantic Ocean (Gartner and Zwerner, 1988). Munday (1976) and Slocombe (1980) described infections in marine mullet (*Mugil cephalus*) and farmed trout (*Salmo trutta*) in Australian waters, while studies in the North Pacific Ocean now show widespread infection in that region. In the north-western Pacific, Gavryuseva (2007) found infection in young coho salmon (*Oncorhynchus kisutch*) in a Kamchatka fish hatchery in Russia, while many species of fish are now known to be affected in the eastern North Pacific up to Alaska (Eaton *et al.*, 1991; Kocan *et al.*, 1999, 2004; Jones and Dawe, 2002). Records of *Ichthyophonus* from Africa and African fish include from *M. cephalus*, in Kowi lagoon (brackish water), south-eastern Cape, South Africa (Paperna, 1986) and from aquarium-held jewel cichlid (*Hemichromis bimaculatus*) (Chauvier, 1979). The low number of records from tropical waters, compared with the many reports from the North Atlantic (east and west), north-east Pacific and the Sea of Japan, probably reflects the research efforts on fish diseases in these areas, rather than a true difference in the pattern of distribution. It can now be concluded safely that the disease has a global occurrence in seawater. Consequently, the recording of new geographical records of the infection is unlikely to be of scientific significance.

The available literature suggests that *Ichthyophonus* is primarily of marine origin, as most reports are from the open sea, estuaries or fish migrating between fresh and marine waters. Many isolations from freshwater fish have been linked directly to the sea, as they have occurred in fish farms using fresh marine fish as feed (Neresheimer and Clodi, 1914; Rucker and Gustafson, 1953; Dorier and Degrange, 1960; Fijan and Maran, 1976; Munday, 1976; Miyazaki and Kubota, 1977a; Slocombe, 1980) or have been from migratory fish entering from the sea. Contradictory to the hypothesis of a solely marine origin of *Ichthyophonus* was the report by Reichenbach-Klinke and Elkan (1965), which listed 48 freshwater fish species among the 83 species reported as infected. However, with the disease being so widespread in coastal marine waters, the lack of evidence of natural epizootics of *Ichthyophonus* in freshwater environments is difficult to explain. The organism is capable of horizontal transmission between fish in fresh water by contact or feeding on infected dead fish (Rucker and Gustafson, 1953;

Gustafson and Rucker, 1956; McVicar and McLay, 1985), but there is no evidence of it becoming established in the vicinity of freshwater fish farms that have become infected or from infected wild fish. Migratory salmonids are known to harbour *Ichthyophonus*, the infection being found in wild sea trout (Robertson, 1909) and regularly in Atlantic salmon (*S. salar*) returning to rivers in Scotland (McVicar, 1982). There is no evidence from extensive disease studies in Scotland that *Ichthyophonus* infection is present in salmonid freshwater populations. A similar conclusion was reached from a comprehensive investigation by Kocan *et al.* (2003, 2004) of the occurrence of *Ichthyophonus* in Yukon River Chinook salmon (*O. tshawytscha*). This showed that when adult salmon entered the river in June, they were already infected at a prevalence level of 25–35%. This level remained relatively stable in the first 1200 miles upstream before increasing as pre-patent infections became detectable and subsequently decreasing 1700 miles upstream. These conclusions were supported in experimental studies (Kocan *et al.*, 1999; Jones and Dawe, 2002) which showed *Ichthyophonus* infected Chinook salmon developed signs of disease 25–35 days following exposure, with mortality occurring between 25 and 60 days post-exposure. The source of the natural infection was not determined, but the possibility of a marine origin was considered. Herring (*Clupea pallasi*) south of the Aleutian Islands were infected heavily, but no infected herring were found north of this. *Ichthyophonus* was not found in pike, grayling, sheefish and juvenile salmon in the river, suggesting the absence of an established epizootic there. However, chum salmon (*O. keta*) and burbot (*Lota lota*) were infected in the vicinity, possibly from eating infected salmon tissue.

Diagnosis from Gross Observations of Clinical Signs

The ability of observers to detect *Ichthyophonus* from gross signs is linked closely to the level of infection and pathogenicity in the host and the tissue sampled. As this differs substantially between different species, obvious signs of disease may vary considerably between different fish (McVicar and McLay, 1985). As with most disease conditions when pathogenicity is high, several changes can accompany the advanced stages of infection, but those accompanying *Ichthyophonus* infection cannot always be used with confidence. These include behavioural abnormalities and changes associated with organ failure, such as lethargy, emaciation, colour anomalies, fluid accumulation, nervous disorders and an increase in mortality. Such changes are evident in groups of captive fish, but are also discernible in wild populations. McVicar (1979, 1981) showed that infected plaice could be selected from commercial catches by weight loss. External signs including skin roughening ('sandpaper effect') and occasional ulceration permitted Sindermann and Scattergood (1954) to identify infected herring (*C. harengus*) in the western North Atlantic. Rahimian and Thulin (1996) and Hodneland *et al.* (1997) also used similar signs to detect infected herring in the Kattegat and Norwegian Sea areas. The presence of tissue granuloma may have led some researchers to suspect *Ichthyophonus* infection in several species of fish and other aquatic animals. To differentiate *Ichthyophonus* from other granuloma-inducing conditions such as mycobacteriosis, it is highly recommended that field diagnosis from fresh material be supported by evidence of the characteristic germination of the parasite after the death of the host (McVicar, 1982). This germination is also normally evident in histological sections when there has been a delay in fixing tissues after the death of the host.

When infected fish are examined internally, it is common for gross white or cream-coloured nodular lesions 1–5 mm in size to be visible throughout most tissues, although, for unknown reasons, the organs infected most heavily may vary in different species of fish (McVicar and McLay, 1985). For example, in haddock (*Melanogrammus aeglefinus*), the most obvious lesions occur in the white muscle, in herring in the heart and in plaice in the liver and kidney. It is necessary to take account of such differences when determining the prevalence or the

effect of infection. These lesions usually consist largely of the granulomatous reaction surrounding single 'spores', 10–250 μm in diameter, or groups of spores. Where the host cellular reaction is weak, as in plaice, the nodules may comprise almost pure *Ichthyophonus* tissue and, in cases of heavy infections, most of the normal organ tissues may be replaced by the parasite. Where the granulomatous reaction is most effective in containing infection (as with cod), the infection may be tolerated and only the granuloma with no remaining evidence of parasite tissue may be found in most lesions. Sindermann (1990) found that the condition of cod improved during an epizootic of *Ichthyophonus* in the Gulf of Maine, presumably due to them feeding on dead and dying herring, so providing further evidence of how well this host may tolerate infection.

Following two special workshops on *Ichthyophonus* in European herring under the auspices of the International Council for the Exploration of the Seas (ICES) in 1991 and 1993 (Anon., 1991, 1993), it was noted that it was possible to obtain an estimate of the prevalence of infection in a herring population through observation of hearts showing gross lesions. In recent infections, or in small fish, the microscopic examination of tissues was also considered desirable. Without doubt, some infected fish are missed if only gross signs are relied on as a sign of infection. Holst (1994) observed a 10% higher detection level of infected herring by microscopic examination of squash preparations of heart tissue than by macroscopic examination, while Rahimian and Thulin (1996) found four times as many infected herring when using microscopic examination. Discrepancies between the prevalence level of infection in the same population of Norwegian spring spawning herring have been found between Russian researchers using pathological evidence including the presence of 1–10 μm sized spores inside phagocytes (Karaseva *et al.*, 1993, 1995) and Norwegian researchers using gross evidence of spores in the heart and other tissues (Hjeltnes and Skagen, 1992; Hodneland *et al.*, 1997). When the association between gross pathological changes and infection as determined by the most sensitive detection method has been verified properly, the visible signs of abnormality may be used safely for rapidly screening large numbers of fish in infected populations. The sensitivity of detection of *Ichthyophonus* could be increased further using molecular techniques (Whipps *et al.*, 2006). The constraints on the use of complex and resource-demanding techniques limit their practical application in field studies on the prevalence of a disease such as *Ichthyophonus.* Then it has to be accepted that the earliest stages of infection will be difficult or, in practice, impossible to detect accurately. Variability in the accuracy of detection and the prevalence levels of infection being determined can be reduced by selecting a cut-off point where the extent of infection is within the limits of practical detection, such as grossly visible heart lesions. Establishing the relationship between the selected index and the most accurate detection method will always be desirable. Hodneland *et al.* (1997) undertook such a systematic study in Norwegian herring, investigating the infection associated with skin papules, blebs, erosion, ulceration and haemorrhage. They demonstrated a close correlation with the presence of *Ichthyophonus* infection detectable by microscopic examination. For comparative epidemiological studies at the population level, the criteria used to designate a sample as positive should:

- produce statistically consistent results by the same and different observer
- be practical in terms of resources (manpower, time and samples available)
- be acceptable to different observers when data are being taken for comparison from different observers, institutes or countries.

Inevitably, whichever criteria are used, there has to be an acceptance that some infected individuals will be missed. The prevalence levels recorded can therefore only be related to the detection methods used.

Microscopic Diagnosis

The opportunity to distinguish *Ichthyophonus* from similar or related organisms based on the morphological characteristics of the parasite in the tissues of infected hosts has been restricted by the lack of obviously different development stages, such as a sexual phase, the absence of rigid parts and the variation in the size and shape of the same development stages. The morphological variations found in different hosts, or even in different parts of the same host, and in cultures might be significant or could be reflections of interspecific host effects and the nutritional and physiological factors. Spanggaard *et al.* (1995) and Spanggaard and Huss (1996) showed that variable pH, carbon dioxide, glucose availability and salinity affected the growth of the parasite.

Fresh squash preparations of infected organs typically reveal the presence of the normally spherical resting stage ('spore'; size from 10 to 250 μm in diameter). These are usually surrounded by varying amounts of host granulomatous reaction tissues, and the use of phase contrast microscopy is advisable to facilitate distinction of them from other similar structures present in the body of fish. However, because of the lack of specific distinguishing features in the spherical bodies ('resting spores') of *Ichthyophonus*, caution should be exercised in diagnosing the disease solely from the observation of these, particularly when there are limited numbers present in the fish. Difficulty is also encountered with hosts that are more resistant to the disease when there are often few or no traces of the parasite left in granuloma. Overall, it is advisable that additional features which are specifically characteristic of *Ichthyophonus* be found before a final diagnosis is made. McVicar (1982) recommended that the characteristic parasite germination, which occurred after the death of the host, might be used as a specific diagnostic index. Spanggaard and Huss (1996) noted that a rise in the carbon dioxide levels as the host died stimulated *Ichthyophonus* cells to germinate and that growth was unaffected by temperature up to 25°C. At a low pH, Spanggaard *et al.* (1995) showed that germination occurred within 4–5 h. Consequently, the re-examination of suspect squash preparations on microscope slides after a period of more than 5 h at room temperature should provide convincing evidence for or against the presence *of Ichthyophonus*.

Histology of infected tissues provides useful data for the diagnosis of *Ichthyophonus*. Spherical bodies typically predominate. Sections from newly dead fish do not normally show germination of the resting cells, and the occurrence of development ('hyphae') in the illustrations shown in published reports on *Ichthyophonus* probably indicates that the tissues had not been fixed immediately after death of the fish. Cells of the parasite occur singly or, more commonly, in groups, typically surrounded by host reaction. Cells in these groups are usually of varying size, indicating a lack of synchrony in growth or division of the parasite. The smallest cells are bound by unilaminate walls of parasitic origin, but in larger cells the wall becomes multilaminate. The cytoplasm of the individual *Ichthyophonus* cells is normally lightly staining with haematoxylin and eosin (H & E) and structurally uniform in appearance, with an irregular network of cords and fine granular material. The smallest cells found contained a single centrally located nucleus (McVicar and McLay, 1985), but larger cells had varying numbers of nuclei, scattered throughout the cytoplasm. The nuclei have a characteristic orange tincture in H & E staining, are pyriform in outline, have a central prominent nucleolus and occasionally have chromatin on the periphery. The outer walls of *Ichthyophonus* cells have a strong periodic acid-Schiff (PAS) positive staining reaction, which is a useful additional aid in detecting low levels of infection. Transmission and scanning electron microscope studies of *Ichthyophonus* (McVicar and McLay, 1985; Paperna, 1986; Rand, 1990; Spanggaard *et al.*, 1996) have shown many characteristic features of the parasite useful for specific identification in individual cases. It has been shown that the mitochondria of *Ichthyophonus* have tubulovesicular cristae (Spangaard *et al.*,

1996; Rahimian, 1998), making this genus distinct from *Dermocystidium*, which has flat cristae (Pekkarinen *et al.*, 2003). However, the complexity of the preparation and the limited amounts of material which can be examined offer no significant advantages in field or routine diagnosis.

If culture conditions are suitable, prolific growth of *Ichthyophonus* can be obtained on minimum eagle's medium (MEM) (McVicar, 1982; Okamoto *et al.*, 1985; Spanggaard *et al.*, 1994; LaPatra *et al.*, 2008). These studies have revealed details of the life cycle and how these may be influenced by surrounding environmental conditions. As a primary method of diagnosing ichthyophoniasis, culture techniques could provide a useful practical adjunct to gross and microscopic features.

Sampling Methods

The distribution of *Ichthyophonus* is not uniform, either between areas or even within populations of fish. As a consequence, there are serious difficulties in determining the true prevalence of infection in natural populations. In designing a programme to determine the level of infection, these factors, which influence sampling variation, should be taken carefully into account and the approach standardized where possible. It is important to note that a low prevalence of *Ichthyophonus* does not necessarily mean a low incidence, as during and following an epizootic this may reflect the high mortality rate among infected individuals (McVicar, 1990). Mellergaard and Spanggaard (1997) suggested that the high degree of temporal and spatial variations in the prevalence of the disease in herring was due partly to the migration patterns of different host stocks, while McVicar (1979) also noted local variations in infection levels in haddock and plaice, linked to differences in the origins of stocks. A positive correlation with host age was noted in plaice (McVicar, 1979) and in herring (Mellergaard and Spanggaard, 1997), possibly due to the higher feed consumption in the larger fish. Holst (1994) found that *Ichthyophonus* infected herring were over-represented on the margins of schools and in scattered layers of herring, possibly because of decreased swimming ability, both in burst speed and sustained swimming speed. Predation of infected herring may be increased by the impaired condition of herring, non-shoaling and other behavioural changes (Hjeltnes and Skagen, 1992). Mellergaard and Spanggaard (1997) found the prevalence of *Ichthyophonus* to be significantly higher (1.8 times) in research vessel catches from prefixed fishing positions, compared with catches from commercial fishing boats taken mainly from dense concentrations of fish. Hodneland *et al.* (1997) found that the relationship between external signs and infection detected by other methods might vary between samples, probably influenced by different selectivity of fishing gear, herring school size, infection dynamics of the disease organism and the skill of the observer. Similarly different levels of infection were found by Kocan *et al.* (2003) when sampling Yukon River salmon. At the lower reaches of the river, salmon were already infected at a prevalence level of 25–35%, this increasing markedly in the middle river (around 730 miles upstream) as pre-patent infections became detectable, then infection prevalence subsequently decreased to less than 15% 1700 miles upstream. This was consistent with a corresponding increase in levels of clinical disease, probably leading to death of infected fish.

The Disease

The most likely route of infection of a fish with *Ichthyophonus*, confirmed by experimental transmission, is through the intestine (McVicar, 1982). Although other routes, such as through the gills or skin lesions, as suggested by Riddell and Alexander (1911) and Sproston (1944), still cannot be discounted completely, they are considered generally to be of lower significance. As with most diseases, it is likely that the success of the pathogen in infecting a new host, its subsequent spread, its persistence within the body and its pathogenicity are linked

inextricably to many influencing factors, including the general stress level and health status of the fish (Olivier, 2002). Such factors may, in part, account for the noted discrepancies in host records of the disease.

Once within the fish body, *Ichthyophonus* shows all of the characteristics of a typical systemic invader. The infective stages spread passively throughout the body through the blood or lymphatic systems and become lodged in capillary beds. Blood-rich organs therefore typically become a primary location of infection and, in these areas, proliferation of the parasite occurs, leading both to a secondary invasive spread in the immediate vicinity and to a release of further infective stages into the circulatory system. The effect of *Ichthyophonus* on individual fish is due to invasion of vital organs, leading to their malfunction (McVicar and McLay, 1985; Kocan *et al.*, 2006).

As a generality, epizootics of many diseases are fairly common in fish farms, but are found relatively rarely in wild fish populations. This may be attributed partly to the level of adaptation between host and parasite established normally in the natural environment through long association between the two organisms and partly because of the difficulty in detecting highly pathogenic diseases in extensive environments such as the open sea. *Ichthyophonus* is therefore unusual among fish parasitic diseases as there is a long history of epizootics in wild fish populations due to this infection. Cox (1916), Daniel (1933), Sindermann and Scattergood (1954) and Sindermann (1956, 1958, 1963, 1990) reported serious epizootics in herring in the Gulf of St Lawrence and the Gulf of Maine off the eastern coast of North America until the mid-1950s. These epizootics led to mass mortality, with an estimated 50% of mature herring destroyed during the period 1954–1956 (Sindermann, 1958; Tibbo and Graham, 1963). A significant reduction in the size of herring stocks in these areas was attributed directly to the infection (Sindermann, 1990). The absence of records of later epizootics has been linked to the smaller size of the herring stocks after that time (Sindermann and Chenoweth, 1993).

Although *Ichthyophonus* has long been known in various species of marine fish in different parts of the world, and especially in European waters, since at least the beginning of the 20th century, serious epizootics were not reported in that region until the 1990s. This may be attributed partly to the lack of epizootiological research, but is probably due to the difficulty in establishing the extent of the effect of the disease in the affected populations. There is evidence that *Ichthyophonus* has been recognized regularly in haddock in Scottish waters, with infected fish often being rejected by fish processors over the past 100 years (McVicar, 1982), but without reports of serious disease-induced mortality at sea. In order to convert *Ichthyophonus* infection prevalence to incidence and calculate the disease rate in plaice, McVicar (1990) used the period taken for the fish to produce antibodies to the parasite as a time marker for the course of development of the disease in a wild population. An infection prevalence of less than 10% was calculated to cause an annual mortality of over 50% in a wild plaice population north of Scotland. This can be considered an epizootic of significant proportions, but, again, no dead fish were observed by research vessels or fishermen in the area. One reason might be that the predation pressures in the area were sufficiently high to remove moribund and newly dead fish rapidly.

The European situation changed in the early 1990s, when mass mortality of herring was observed for the first time in the Kattegat, with dead fish floating on the surface, on the sea bottom, resulting in clogging of trawls, and washing up on beaches along the Swedish west coast and in the Sound between Denmark and Sweden (Anon., 1991). Further studies revealed high prevalence of infection with *Ichthyophonus*, up to 100% in certain areas of the northern North Sea, and also that the infection was widespread in the Norwegian Sea, Skagerrak, Kattegat and western Baltic Sea. The factors which led to the development of an epizootic of *Ichthyophonus* in herring of the eastern North Atlantic region are unknown, although as indicated above, the parasite has

been present in the area over an extended period, even leading to significant mortality in species such as plaice (McVicar, 1981). It may be significant that the period between the late 1980s and early 1990s was characterized by a reduced fishing pressure and high population numbers of herring in these areas. The possibility of changes in the distribution of herring populations over the same period was also considered as important (Anon., 1993). Sindermann and Scattergood (1954) suggested that temperature or salinity changes in the sea could be influencing factors. The occurrence of the European outbreak led to a resurgence of studies on the parasite through the auspices of ICES (Anon., 1991, 1993) and independently by researchers in Denmark, Norway, Russia, Scotland and Sweden (Hjeltnes and Skagen, 1992; Karaseva *et al.*, 1993, 1995; Rahimian and Thulin, 1996; Hodneland *et al.*, 1997; Mellergaard and Spanggaard, 1997). It has been thus determined that a major epizootic of *Ichthyophonus* occurred in European herring stocks in the early 1990s, covering an extensive area of the western Baltic to the north of Norway and Russian waters. Similarly, populations of herring from Washington State, British Columbia and the Gulf of Alaska have been found to be infected heavily with *Ichthyophonus* (Marty *et al.*, 1998; Hershberger *et al.*, 2002; Jones and Dawe, 2002) and, in Icelandic waters, a major epizootic of *Ichthyophonus* in herring was reported in 2008 in which around 40% of the stock was thought to be infected (Icelandic News in English, December 2008).

From a prevalence of 3.7–10.6% of herring stocks in commercial and research vessel catches in the North Sea in 1991, Mellergaard and Spanggaard (1997) calculated the total *Ichthyophonus* induced mortality of herring to be between 12.8 and 36.8% of the North Sea herring population and referred to data indicating a 25% reduction in the herring spawning-stock biomass over the same period. These authors also referred to a report (ICES, 1996, cited by Mellergaard and Spanggaard, 1997) showing a reduction in the herring spawning-stock biomass from 1.1 Mt in 1991 to 0.5 Mt in 1995, but suggested that this reduction might be due to a combination of changed fishing intensity and the effect of *Ichthyophonus.* Patterson (1996) modelled the outbreak and estimated up to 9.6% of potential herring fishery production was lost due to the fungus *Ichthyophonus* in the worst affected years. The prevalence level of *Ichthyophonus* showed a decreasing trend from 1991 to 1993 to a level below 1% in most areas (Mellergaard and Spanggaard, 1997). This level was considered to represent background non-epizootic levels in the North Sea area, although the infection was known to be present still in herring in several European herring stocks (Anon., 1997). Although other fish species (flounder, sprat) were also found concurrently affected in the area (Rahimian, 1994), no similar mortality was shown during the epizootic in herring. Periodic outbreaks of *Ichthyophonus* continue to be detected in other areas. Epizootics have also been detected in Pacific salmon in the Yukon River (Kocan *et al.*, 2003), with an adverse impact on spawning success.

Although records of infection may suggest an infectious agent with a high level of transmission and infection success and a high pathogenicity, it cannot be assumed that infection with *Ichthyophonus* will lead inevitably to a systemic infection with overt disease or the beginning of an epizootic. There are many records of the parasite occurring without accompanying evidence of serious consequences to individual fish or to populations. McVicar and McLay (1985) gave evidence that different species of fish, or even different individuals of the same species, might show a range of resistance or tolerance of the infection. Species such as plaice and herring are highly susceptible, with intermediate tolerance in haddock and rainbow trout (*O. mykiss*), while other species show a high level of resistance. Some species of fish such as cod (*Gadus morhua*) may be more refractory than others (McVicar, 1982; McVicar and McLay, 1985). Others, such as goldfish (*Carassius auratus*), guppy (*Lebistes reticulates*), squawfish (*Ptychocheilus oregonensis*) and catfish (*Ameiurus nebulosus*) are resistant in experimental challenge (Gustafson and Rucker, 1956). Even in known susceptible species,

infection of a new host is not necessarily a simple process of a susceptible host coming into contact with an infective stage of the parasite (Olivier, 2002). Sindermann (1965) reported that multiple, massive exposures on successive days with *Ichthyophonus* might be necessary to obtain infection, as in herring.

During an epizootic of the disease in the Gulf of St Lawrence with high mortality in populations of herring, alewives (*Alosa pseudoharengus*) and mackerel (*Scomber scombrus*), Sindermann (1966) noted that cod in that area benefited from the disease and showed an increased growth rate. This probably was related to the abundance of feed in the form of diseased, dying and dead fish. There is a contradiction in the literature regarding the effect of the disease on cod in European waters. McVicar (1982) showed a strong host cellular-containment reaction to the infection in this species. In cod populations off the Scottish coast, while infection could be detected in some fish, none showed an extensive invasion (A.H. McVicar, unpublished observations). In contrast, Moller (1974) found 15.2% of cod in the western Baltic Sea infected with *Ichthyosporidium* (*Ichthyophonus*) *hoferi*. Kock (1975) suggested that most of the cod mortality in a cage experiment had an associated *Ichthyophonus* infection and by implication suggested that this was the main cause of death. To clarify this apparent contradiction, further studies are necessary in cod, for example on both the strains of parasite and fish stocks, particularly in view of the interest in the culture of this species in fish farms. In plaice, the disease is considered lethal in approximately 2 months (McVicar, 1990). Because of the high mortality rate, the prevalence found in the infected population was consistently low (less than 10%). A similarly rapid removal of infected fish from the area might explain why Rahimian (1994) did not find infected plaice in the Kattegat during the major epizootic of the disease in herring in the early 1990s. In herring, the disease may progress either through an acute course, with death occurring within 1 month of exposure, or through a chronic course, with some fish surviving for up to 18 months (Sindermann, 1956, 1958). Sindermann and Chenoweth (1993) hypothesized that the low prevalence of *Ichthyophonus* observed during and following an epizootic simply might reflect a high mortality rate of infected individuals. A similar decline in infection prevalence has been observed in *Ichthyophonus* infection in several species of fish in the North Atlantic during and after epizootics (McVicar, 1990). The implications of *Ichthyophonus* outbreaks to fisheries stock management have been considered by Patterson (1996). Similarly, *Ichthyophonus* infection has been identified by Deriso *et al.* (2008) as a possible secondary factor in the collapse and subsequent lack of recovery of the Pacific herring stock that spawns in Prince William Sound. They considered the implication of the parasite to fisheries management and noted that that it might be difficult simultaneously to increase the production of pink salmon and maintain a viable Pacific herring fishery. The impact can be extended to other commercially important fisheries, and a whole ecosystem approach may be needed to evaluate the costs and benefits.

The introduction of *Ichthyophonus* into a freshwater rainbow trout farm can have serious consequences. Rucker and Gustafson (1953) recorded the death of 50–90% of stocks on one farm in the USA or limited effects, as noted by McVicar (1982), on a rainbow trout farm in Scotland. Aspects of the host's response, particularly the cellular reaction, have been implicated in the intra- and interspecific variations in fish susceptibility to *Ichthyophonus.*

The Microorganism

The taxonomic position of *Ichthyophonus* has had a chequered past. Research interest on fish diseases increased considerably around the turn of the 20th century, and diseases with clearly visible gross signs, such as ichthyophoniasis, frequently became the subject of study. The standard of observation in the early studies was exceptionally high and they gave sufficient

details in text and accompanying drawings for later comparative studies. However, communication was difficult during that period and the publication of the studies often in journals with limited circulation tended to lead to a lack of cross-referencing. Much of the subsequent confusion regarding the nomenclature used for the infective agent originates from this period and may, at least in part, be attributed to these difficulties.

The disease was first reported by von Hofer (1893) as dizziness disease (*Taumelkrankheit*) in trout and salmon in Germany. This and other early studies described and illustrated the morphology and some stages of the development of the infective organism in sufficient detail to demonstrate that the same or closely related organism was clearly being examined. Johnstone (1905) described an epizootic in a tank population of plaice, in the Isle of Man, and, on the basis of morphology, tentatively linked the infective agent with fungi of the Entomophthorineae. Robertson (1909) reported what appeared to have been the same disease from sea trout (*S. trutta*), flounder (*Platichthys flesus*) and haddock. Because it was thought that the parasite had some similarities to a protozoan described by Caullery and Mesnil (1905), the parasite was described as *Ichthyosporidium gastrophilum*. Laveran and Pettit (1910) recorded the same disease causing high mortality in a fish farm in France. They noted that the normal means of propagation of the parasite might be by subdivision of primary protoplasmic bodies into secondary cysts and believed that it had some affinities with the protozoa, especially the Haplosporidia, but considered that there were some plant-like structures present. From material obtained from two infected rainbow trout, Plehn and Mulsow (1911) finally described the organism causing the disease as *I. hoferi*, classing it in the Phycomycetes close to the Chytridinae. Almost simultaneously, in a study of diseased haddock (known as 'spotted haddock', 'greasers' or 'smelly haddock') caught off the west of Scotland in 1911, Williamson (1913) illustrated clearly the same disease signs and morphological features, and considered the parasite as undescribed. Some points of resemblance to stages in certain Myxo-sporidia (*sic*) were found and the name *Dokus adus* proposed. However, under the rules of nomenclature, this name was a junior synonym and became redundant. Pettit (1913) recognized that the first use of the generic name in relation to the disease agent had been by Caullery and Mesnil (1905) as *Ichthyosporidium* and, also according to nomenclature rules, transferred *Ichthyophonus hoferi* to *Ichthyosporidium hoferi*. This was accepted by some but not all researchers and the disease appeared regularly in the subsequent literature under both generic names, creating considerable confusion. However, in a review of the taxonomy of *Ichthyosporidium*, Sprague (1965) concluded that this genus should be reserved for a protozoan group, with *I. giganteum* as the type species of the genus. As *Ichthyosporidium hoferi* clearly was considered to be unrelated to that type species, Sprague changed the name back to that of the first valid named description of the organism, namely *Ichthyophonus hoferi* Plehn and Mulsow (1911). *Ichthyophonus* is now generally considered the valid generic name of the parasite.

The confusion regarding the taxonomy of the causative agent of ichthyophoniasis is not restricted to the genus level. The original description of *I. hoferi* by Plehn and Mulsow (1911) was incomplete and consequently led to difficulties when direct comparisons were attempted. In addition, only vegetative growth stages were observed, both *in vitro* and *in vivo* (Spanggaard *et al.*, 1995). As these stages were highly malleable in their morphological appearance, depending on host and substrate conditions, the stages available for specific comparison and identification were limited and variable (McVicar, 1982; Spanggaard *et al.*, 1995). Spanggaard and Huss (1996) demonstrated that different types of development of *Ichthyophonus* could be triggered by changes in pH, temperature, salinity and carbon dioxide tension. Rand (1994) also described an unusual form of *Ichthyophonus* in yellowtail flounder (*Limanda ferruginea*), which bore similarities to atypical features noted in other isolations (Jeeps, 1937; Hendricks, 1972; Gartner and Zwerner, 1988). He raised the possibility that these

could be a separate 'entity' from the 'species' *I. hoferi* as commonly described, but cautioned against the erection of new species, due to the then lack of detailed taxonomic knowledge of *I. hoferi*. Alderman (1976, 1982) suggested that *I. hoferi* had been used to describe a complex of organisms and had become a 'wastebasket' taxon, with poorly defined species limits. It is unlikely that the specific or strain relationships among all the different isolates of *Ichthyophonus* will be resolved until samples from the original sources are studied with new approaches that combine structural, biochemical and molecular data, such as that used by Ragan *et al*. (1996) and Spanggaard *et al*. (1996).

The lower fungi, lower algae and lower protozoans show many common features and, not surprisingly, the taxonomy of these organisms has been the subject of controversy (Alderman, 1976; Lom and Dykova, 1992). Also, many researchers who have worked on these organisms are pathologists and diagnosticians and not specialist taxonomists, and it is not surprising that there have been few in-depth assessments of the taxonomy. Many researchers either have chosen to follow the most recent trend or have grouped the organism on the basis of the most obvious morphological features. Consequently, *Ichthyophonus*, *Dermocystidium* and similar organisms remained largely as taxonomic orphans, which were assigned variously to the protozoans and fungi, because of their superficial morphological similarities to these groups, without strong conviction being shown by most authors. The absence of rigid body parts, which could be useful in diagnosis, and the typically high level of variability in the structure of the life-cycle stages available for study, together with the absence of definitive sexual stages, undoubtedly have also contributed to the practice of using genera or even species as a convenient 'wastebasket' for partially defined organisms (Alderman, 1982). Through the use of ultrastructural features, several workers (Alderman *et al*., 1974; Corliss, 1987) have attempted to resolve the difficulties in the phylogenetic placing of these organisms, but, lacking definitive stages, serious questions have remained as to their exact relationships. For many years, it appeared to have been generally accepted that, until more definitive evidence was available, the suggestion made by Alderman (1982) to place *Ichthyophonus* in the Fungi *incertae sedis* should be followed.

With the application of molecular biological techniques to the diagnosis and differentiation of fish diseases, the opportunity was taken to re-evaluate the position of *Ichthyophonus*, *Dermocystidium* and similar organisms. Ragan *et al*. (1996) and Spanggaard *et al*. (1996) used amplification and sequencing of small subunit ribosomal ribonucleic acid (RNA) in these and similar organisms (e.g. 'rosette agent'). These two groups of researchers independently reached the conclusion that *Ichthyophonus* was a member of a lower protistan group (the DRIPs clade) incorporating the *Dermocystidium*, rosette agent, *Ichthyophonus* and *Psorospermium* groups of organisms, situated near to the choanoflagellates and not members of the Fungi. Some inconsistencies in this grouping were noted, particularly when using ultrastructural and biochemical information (Spanggaard *et al*., 1996). The DRIP clade was later extended by Cavalier-Smith (1998) to Ichthyosporea in the subphylum Choanozoa, representing a group of organisms near the divergence of animals and fungi. Mendoza *et al*. (2002) reviewed the morphological features, pathogenic roles and phylogenetic relationships of the class Mesomycetozoa and noted the distinction between the Dermocystida, which mostly produced uniflagellate zoospores, and the Ichthyophonida species, which mostly produced amoeba-like cells. Pekkarinen *et al*. (2003) pointed out that mitochondrial cristae had often been considered as a guide to the phylogenetic position of organisms and noted that *Ichthyophonus* had tubulovesicular cristae distinct from the *Dermocystidium* group with flat mitochondrial cristae. However, as other organisms currently included in the *Ichthyophonus* group had been found to have vesicular or mostly flat cristae, they considered that the phylogenetic grouping using the nature of mitochondrial cristae was not unambiguous. With their

sequencing studies, Ragan *et al.* (2003) could not resolve whether the *Ichthyophonus* they studied was a specific relative of animals or of fungi, but found features previously reported among fungi only. Steenkamp *et al.* (2006) concluded that *Ichthyophonus* was an animal parasite and that the Ichthyosporeans formed a group which, together with the ministeriid and choanoflagellates, made up two to three separate sister lineages to animals. They suggested that the eukaryotic supergroup, Opisthokonta, including animals and fungi, should be expanded to include a diverse collection of primitively single-celled eukaryotes previously classified as Protozoa, including, among others, the ichthyosporeans.

Variation within the genus *Ichthyophonus* has become increasingly apparent. Hershberger *et al.* (2008) demonstrated that spores from marine hosts had a greater seawater tolerance than spores from freshwater hosts and vice versa, indicating an adaptation to the host environment. Using sequencing techniques, Criscione *et al.* (2002) detected ribosomal DNA sequences which indicated isolated populations of *I. hoferi* in the north-eastern Pacific Ocean. Similarly, Halos *et al.* (2005) found that the genomic sequences of *Ichthyophonus* isolates obtained from Puget Sound rockfish, Pacific herring and Yukon River Chinook salmon were identical in the sequences studied, but were different from *Ichthyophonus* sequences previously isolated from four different species of rockfish from the north-eastern Pacific Ocean. Rand (1994) noted differences in *I. hoferi* isolations from the yellowtail flounder (*L. ferruginea*) from the Nova Scotia shelf which, with additional evidence, Rand *et al.* (2000) considered sufficient to erect a new species, *I. irregularis*. Evidently, much further clarification of the exact taxonomic position of *Ichthyophonus*, variation within the genus and comparison of isolations from different host groups are still required.

Life Cycle

There have been many investigations on parts of the life cycle *of Ichthyophonus* through studies on various fish species with natural and experimental infections and through *in vitro* culture. Only Sproston (1944) described a range of complex structures and an intricate life cycle, including sexual reproduction. The uniqueness of this report among the many studies on the disease places some doubt on its validity, and it is possible that a mixed infection of more than one organism is present. As a whole, other studies have shown a limited range of morphological structures, with there being general agreement that the life cycle is relatively simple (Daniel, 1933; Fish, 1934; Sindermann and Scattergood, 1954; Dorier and Degrange, 1960; Miyazaki and Kubota, 1977b; Chien *et al.*, 1979a,b; McVicar, 1982; Okamoto *et al.*, 1985; Spanggaard *et al.*, 1995; LaPatra *et al.*, 2008).

The most commonly observed stage of the parasite is the spherical cell (often called the 'resting spore') (Fig. 19.1), characterized by a thick wall, surrounded to a greater or lesser extent by a capsule of host reaction tissue. There are many light microscopic descriptions of this stage in the literature, for example, by Sproston (1944), Sindermann and Scattergood (1954), McVicar (1982) and Rand (1990). Although some variations have been noted in the size of the parasite in different organs and different host species, there is a remarkable consistency in the structure. When the spherical cell of *Ichthyophonus* has been subjected to detailed electron microscopic study (McVicar and McLay, 1985; Paperna, 1986; Rand, 1990; Spanggaard *et al.*, 1995), there again has been general agreement among the findings. These have shown a cell wall, of varying thickness, which is fibrillar and densely laminated in structure, a fine granular cytoplasm packed with ribosomes, scattered vesicles, some with lipid-like contents, mitochondria with tubulovesicular cristae and a varying number of nuclei. Despite the large number of nuclei in many cells, there was no evidence of zonation of the cytoplasm into functional areas or partitioning of the cytoplasm between nuclei, which could suggest cellular division. The nuclei were spherical, each with a prominent central spherical nucleolus.

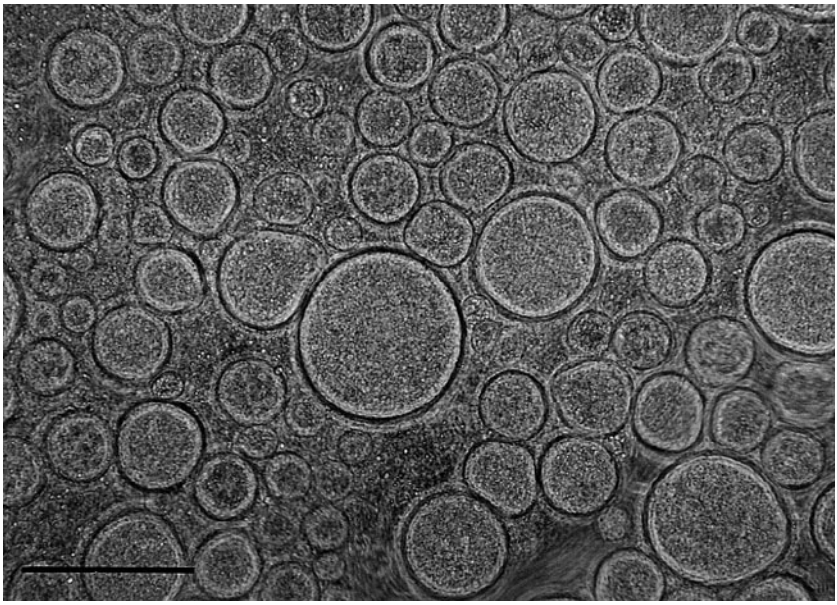
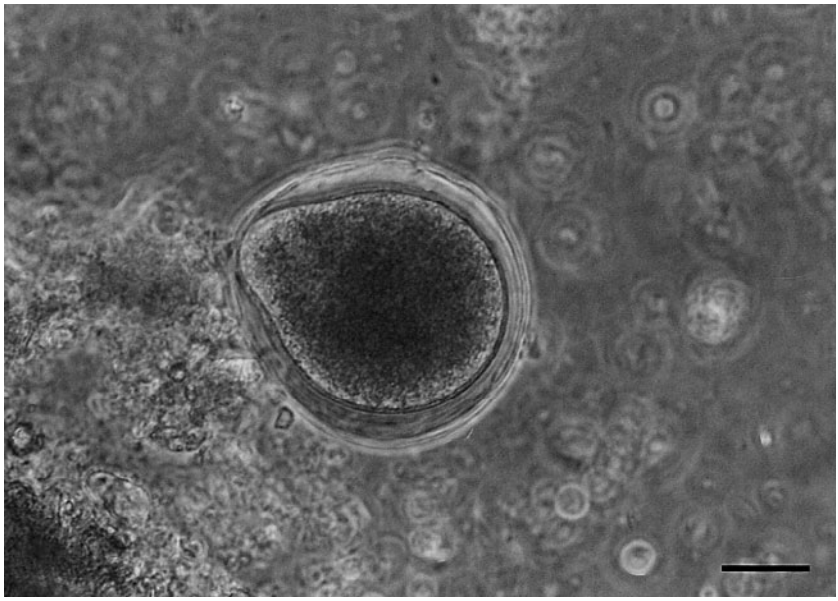
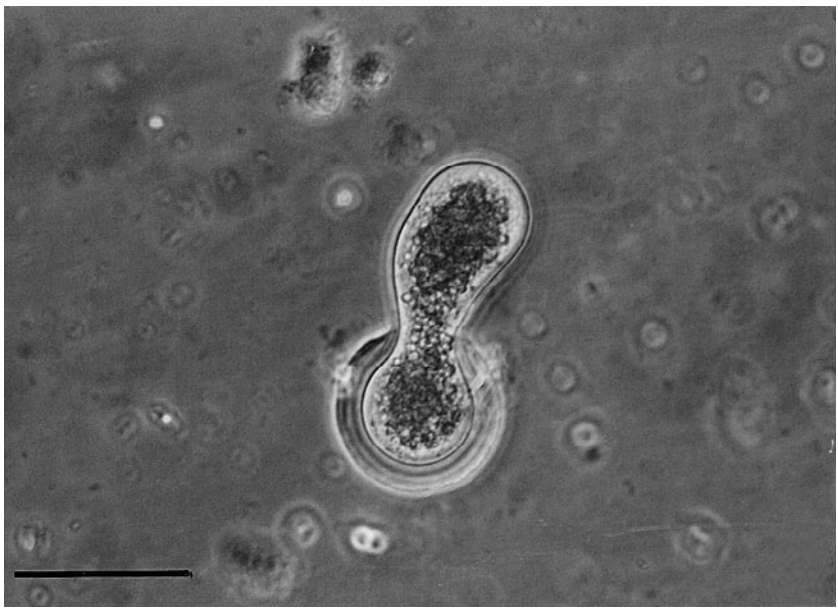


Fig. 19.1. Squash preparation of plaice liver infected with *Ichthyophonus*. Scale bar, 100 µm.

The typical initial infective challenge is generally considered to be through the ingestion of thick-walled resting spores with infected feed (especially fish) or directly from water. Jeeps (1937) recorded infection in copepods, and *Calanus finmarchicus* could be infected experimentally (Sindermann and Scattergood, 1954). However, it is possible that these may act as paratenic hosts, which accumulate or harbour temporary infections, so concentrating infective stages for plankton-feeding fish. With the possibility that this infection when occurring naturally is a *Calanus*-specific pathogen (Torgersen *et al.*, 2002), a re-evaluation of these observations would be appropriate. Several studies (Sindermann and Scattergood, 1954; Sindermann, 1958) have found that a large challenge of fish with infective material or a repeated challenge may be necessary for infection to become established. Dorier and Degrange (1960) suggested that this might be related to the time taken for spores to develop in relation to the time taken for meals of different sizes to pass through the fish intestine. This interpretation has been challenged subsequently as the parasite has been shown to pass rapidly through its development stages in 3–5 h under conditions of varying pH (Spanggaard *et al.*, 1994, 1995) and single peroral infections have proved to be successful in various species of fish (McVicar, 1982). Raffel *et al.* (2006) suggested that leeches might be involved in the transmission of *Ichthyophonus*-like infections in amphibians.

Germination of ingested spores in the host intestine typically involves the development of tubular protrusions (often called germination tubes or 'hyphae') (Figs 19.2 and 19.3), which usually become branched (Fig. 19.4). These are similar to those observed in tissues when the host dies (McVicar, 1982; Spanggaard *et al.*, 1994, 1995). Although such germination features are structurally similar, there may be subtle differences in the nature of such hyphae under different conditions of growth. Within the fish stomach, the stimulation of 'resting spores' to develop may be related especially to the low or changing pH and to other factors in the environment, such as glucose (Spanggaard *et al.*, 1994, 1995). But other factors were thought to be involved in the post-mortem





Figs 19.2 and 19.3. Beginning of germination of *Ichthyophonus* spherical bodies in the intestine of an experimentally challenged rainbow trout 4–6 h after feeding. Scale bar = 100 µm.

development in dead fish tissues, as the pH there did not become sufficiently low to trigger hyphal development. Spanggaard *et al.* (1995) used scanning electron microscopy to visualize the morphology of the developmental stages of *Ichthyophonus* cultured *in vitro* and this showed the surface of the spherical cell and developing germination tube to have a generally smooth wall. An indication of a longitudinal 'keel' along

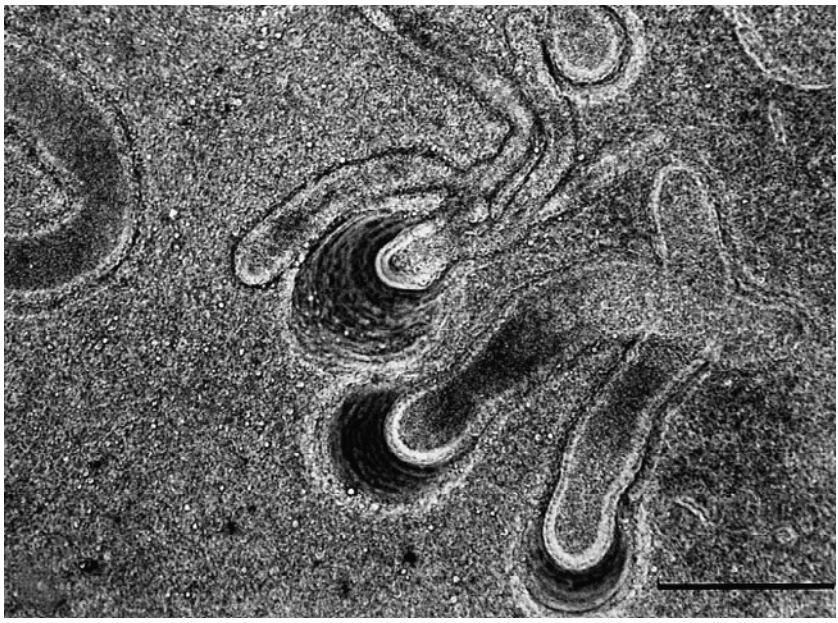


Fig. 19.4. Development of branched germination tubes ('hyphae') from spherical bodies of *Ichthyophonus* in the intestine of an experimentally infected rainbow trout. Scale bar, 100 µm.

the length of the latter was also shown in one instance.

Development of the parasite typically proceeds by the cytoplasmic contents of the spore flowing up the germination tubes ('hyphae') to accumulate in the multiple tips, with subsequent rounding up and walling off to form thin-walled spherical structures. These are of varying size, frequently detach from their parent structures and may be scattered individually around the original infection site or more distally throughout the body. The outcome of this proliferation in the tissues of a dead host or in the gut of a potential new host which has eaten live infected prey would, in both cases, result in a large increase in the number of potentially infective units. There are significant advantages to proliferate potentially invasive stages at the point where transmission to new hosts is occurring.

Further proliferation of the infection occurs through the subdivision of the contents of each of the spherical bodies (Figs 19.5 and 19.6), ultimately to produce small uninucleate stages (Dorier and Degrange, 1960; McVicar, 1982; Okamoto *et al.*, 1985; Spanggaard *et al.*, 1995). These may show intermittent rotational movements while enclosed within the wall of the spherical body and, when released, they have the power of movement (probably amoeboid) away from the point of their release (McVicar, 1982). Various studies have suggested that these amoeboid bodies could survive from 1 to 5 days after release, and it has been suggested that these bodies ('endospores') could be associated with the infective stage of *Ichthyophonus*. Because of their small size, they will be difficult to detect in living fish, particularly in natural infections, and this has led to speculation on the precise route of infection. However, in experimental situations with exceptionally large infective doses, transmission electron microscopy has provided some clarification (A.H. McVicar and J. Gilmour, unpublished results). Small membrane-bound bodies with cytoplasmic contents typical of *Ichthyophonus* were observed adhering to the glycocalyx layer of the brush border of the intestinal mucosal cells.

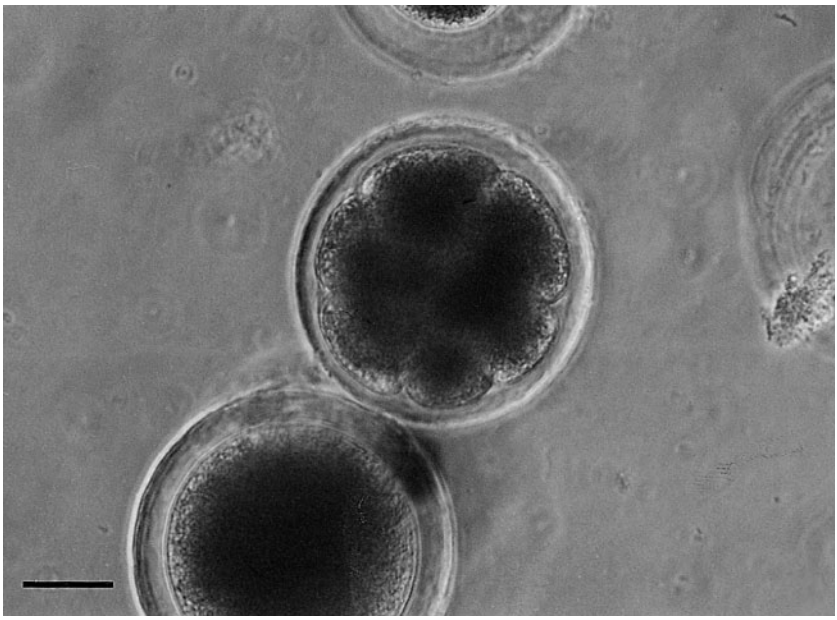


Fig.19.5. Internal subdivision of the cytoplasmic contents of a spherical body produced at the tip of a germination tube of *Ichthyophonus*. Scale bar, 100 µm.

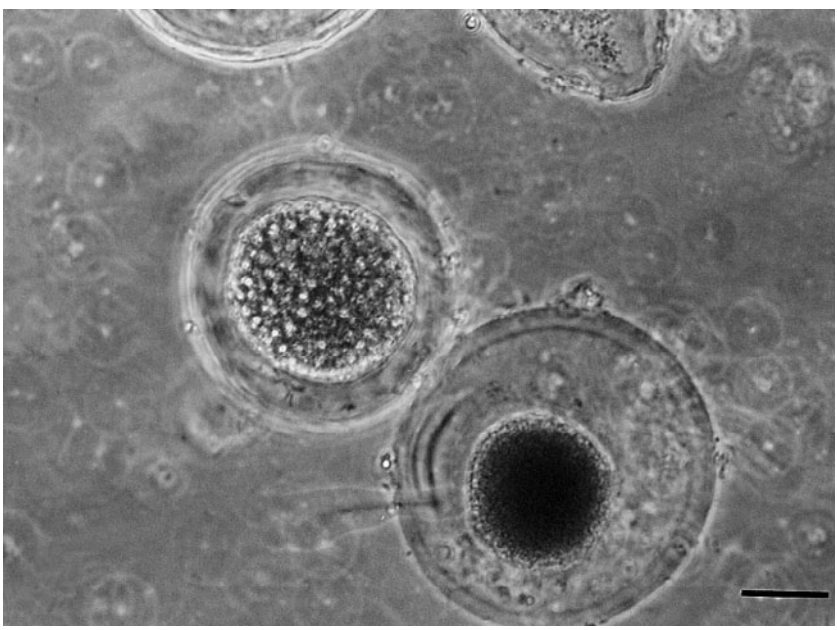


Fig. 19.6. Spherical body with uninucleate motile bodies in the intestine of a rainbow trout experimentally challenged with *Ichthyophonus* approximately 5 h after feeding. Scale bar, 100 µm.

Slender 'hyphae' were found penetrating between the microvilli of the brush border and, finally, these hyphae were detected penetrating through the cytoplasm of the mucosal cell (Fig. 19.7) from the cell surface. No penetration was observed through the intercellular spaces between mucosal cells, presumably due to the greater difficulty in breaking through the tight junction between the cells. Similar 'hyphae' were detected deep in the cytoplasm of mucosal cells and, although none were found penetrating through the basement membrane, it was likely that this would not be an impenetrable barrier for further passage to the intercellular lymphatic spaces of the gut wall.

Once inside the fluid spaces of the host tissues, the subsequent spread of small infective units throughout the body via the circulatory system may then occur. The predilection of infection for blood-rich tissues in most fish infections indicates the importance of the host circulatory system in the dissemination of the infection within the host. Rapid growth and nuclear proliferation of the invasive stage to form typical spherical bodies in the tissues of fish have been reported (Dorier and Degrange, 1960), with similar rapid growth also found in culture conditions (McVicar, 1982; Spanggaard *et al.*, 1994). In each case, this leads ultimately to the further subdivision of the spores and the formation of new infective elements. Dorier and Degrange (1960) suggested that a secondary invasion might occur within about 8 days of a primary infection. The information currently available indicates that production of germination tubes, such as is observed in the tissues of a dead host or in the intestinal contents after an infective meal, does not normally occur in the tissues of the living host. In the latter, the infective stages are released into

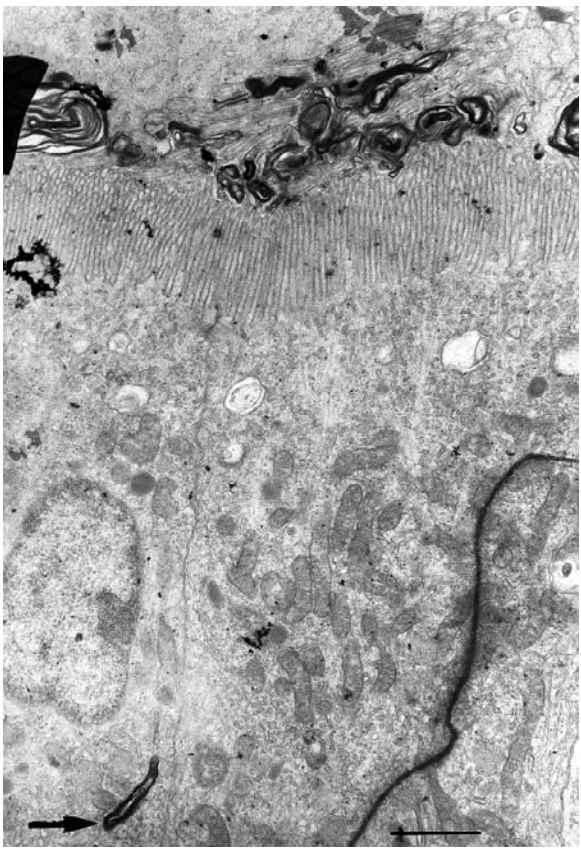


Fig. 19.7. Electron-microscope (EM) section through the microvillous border of the intestine of a rainbow trout experimentally challenged with *Ichthyophonus*. *Ichthyophonus*-like bodies in the gut contents are applied to the tips of the microvilli and a germination tube ('hypha') is penetrating through a mucosal cell (arrowed). Scale bar, 2 μm.

the circulatory system or into adjacent tissues by the rupture of multinuclear bodies. Such differences may be related to pH and availability of nutrients and could be an adaptation to facilitate infection, as suggested by Spanggaard *et al.* (1994).

With the possible exception of the study by Sproston (1944), which, as indicated above, has some unresolved uncertainties about it, no research on *Ichthyophonus* has reported a sexual or conjugative phase as part of the life cycle. This paucity of information would suggest either that such events do not occur as a regular event in the life cycle, the stages found being part of a purely vegetative proliferation, or that conjugation occurs in a form which is rapid and not obvious. More detailed study of the small and motile 'amoeboid' stages seems to be justified, as these characteristics are common for the gamete stages in many other Ichthyosporeans.

Release of infective stages has been noted through the gills and intestinal mucosa (Dorier and Degrange, 1960), through necrotic areas of the skin (Sindermann and Scattergood, 1954) or through kidney tubules (McVicar, 1982), but it is probable that the main route of transmission of infection between hosts is through susceptible potential hosts feeding on moribund or dead hosts. Various authors (Jeeps, 1937; Sindermann and Scattergood, 1954) have raised the possibility of paratenic or transport hosts such as copepods being involved, particularly for pelagic species such as herring, where the main dietary component is plankton.

Control and Treatment

The widespread natural reservoir of infection in marine and estuarine waters and the relative ease of horizontal experimental transmission of the disease between most species of fish could suggest that wild and farmed fish stocks in these areas would be at risk from the infection. However, in practice, there have been no records of farmed stocks becoming infected because of the proximity of infected wild stocks, as in European (McVicar, 1990) or in Canadian waters (McVicar *et al.*, 2006), areas where epizootics have occurred in wild fish populations. This is despite the size of the Atlantic salmon sea cage farming industry in Europe and Canada and the yellowtail industry in Japan in regions known to harbour high levels of *Ichthyophonus* infection. Sindermann and Scattergood (1954) have found that repeated exposure of herring to large doses of *Ichthyophonus* is necessary to establish experimental infection, and it is possible that similar difficulties may also occur in nature during non-epizootic episodes if infective stages are disseminated sufficiently in the aquatic environment before encountering a susceptible host.

An assessment of the literature indicates that for many fish species infection is acute, with associated high pathogenicity. With these, several researchers have considered the disease to be inevitably terminal. Van Duijn (1956) noted that it was impossible to heal fish infected with *Ichthyophonus*, but suggested that fungicidal drugs, such as phoxethol, could be partially effective against the early stages of the infection. Although ichthyophoniasis may often have major economic consequences when occurring in fish farms and aquaria, since that report there have been no significant research efforts on chemotherapy of the disease. With this absence of generally recommended treatment methods, efforts are currently better focused on prevention of the disease rather than its cure.

Infections in both freshwater and marine farms have been commonly associated with the use by fish farmers of fresh marine fish as feed (Pettit, 1913; Sindermann and Scattergood, 1954; McVicar, 1979; Slocombe, 1980; Okamoto *et al.*, 1985). A particular feature of the life cycle of *Ichthyophonus* is the massive replication of the parasite after the death of the host, possibly stimulated by the change in pH, change in carbon dioxide tension (Spanggaard and Huss, 1996) or loss of the integrity of the host cellular defences (McVicar and McLay, 1985). The consequence is a major increase in the number of infective stages being available. Using as feed, material from *Ichthyophonus* infected fish which have been dead for a prolonged period will therefore

pose a particularly high risk. Special attention should be paid to avoiding this source of infection in fish farms. In view of the length of time this association has been known (e.g. by Plehn and Mulsow, 1911; Pettit, 1913; Rucker and Gustafson, 1953), it is disappointing that infections from using such practices still occur regularly in fish farms. The spherical bodies ('resting spores') of *Ichthyophonus* are resilient to a variety of physical conditions, a feature which could pose particular problems in avoidance, prevention and treatment of the disease in cultured or aquarium fish. Spores have been shown to survive for almost 2 years in seawater (Spanggaard and Huss, 1996) and to be fully viable after 6 months in sterile seawater (Sindermann and Scattergood, 1954). Spanggaard and Huss (1996) also showed that the parasite could grow well in the temperature range 0–25°C and between pH 3 and 7 (3–9 was reported by Okamoto *et al*., 1985) but was not able to tolerate salinities above 4% sodium chloride (NaCl). Most agents infectious to fish can be destroyed by treatment of infected tissues with high or low temperatures. Slocombe (1980) found *Ichthyophonus* infection to persist after 2 days at –8°C, while Athanassopoulou (1992) found that the parasite was killed at –20°C. Gustafson and Rucker (1956) suggested that freezing might be a suitable method of killing infection in wild fish being used as a feed source in fish farms. Spanggaard and Huss (1996) demonstrated experimentally a maximum survival temperature of 40°C for a period of 3 min. As low temperatures are being used increasingly in the processing of compounded foods for use in aquaculture industries, some caution should be exercised when marine fish are being used as a source of raw material. However, the absence so far of any infection associated with this feed currently indicates a low risk from this area.

Host Response

Ichthyophonus is highly antigenic and elicits a strong host cellular reaction and, at least in some species of fish, a marked humoral response (McVicar, 1982). Using an indirect fluorescent antibody test on germinating spores in culture (incubated with serum from an infected fish, rabbit antibody against antibody of that fish species and goat anti-rabbit antibody), it has been shown that the general surface of the parasite shows a positive reaction. A particularly strong reaction occurs in a cap surrounding the tips of the developing germination tubes (Fig. 19.8). The nature of the antigen in this area has not been characterized, but may be an enzymatic system associated with the ability of the hyphae to penetrate through host tissue and, in particular, the surrounding capsule of host reaction after the death of the fish. However, Spanggaard (1996) found no or only slight enzyme activity associated with the growth of *Ichthyophonus* in culture, a surprising result in view of the characteristic softness and slimy texture of infected herring fillets. However, a very high protease activity was found associated with *I. hoferi* infection in Alaska pollock by Kimura *et al*. (2004) in their application for US patent 6821546. The development of a strong precipitating antibody reaction in infected fish (plaice, turbot (*Psetta maxima*) and rainbow trout) was demonstrated by McVicar (1982) in natural and experimental infections of *Ichthyophonus*. The development of antibody in all experimentally infected plaice and the absence of antibody in all uninfected plaice in the wild indicated that antibody production was always a consequence of *Ichthyophonus* infection in this species (McVicar, 1982, 1990). However, not all naturally infected plaice had detectable antibody to *Ichthyophonus*. This result was attributed to the duration of the infection being too short for the fish to respond to the infection, a proposal supported by results from plaice in experimental infections where antibody could be detected in all infected fish after approximately 30 days (McVicar, 1990). Despite the strong antibody reaction against *Ichthyophonus*, there was no evidence that this conferred any protection on plaice and it was concluded that the infection was invariably lethal in this species. A similar conclusion that the disease often has severe effects on the viability of or is progressive to terminal in herring has

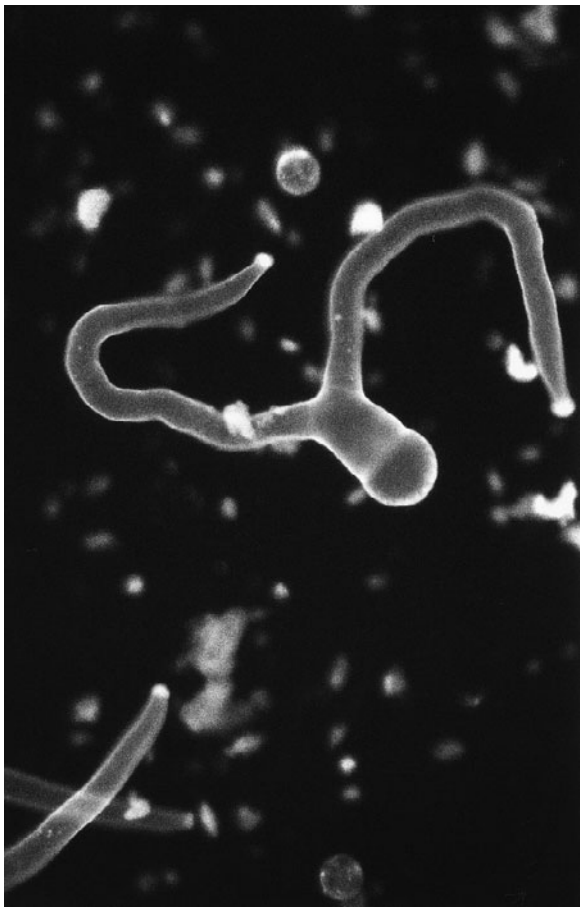


Fig. 19.8. Germinating *Ichthyophonus* spherical cell in MEM culture tested by an indirect fluorescent antibody test (IFAT) for the presence of host-stimulating antigen. The exterior surface of the tips of the growing germination tubes shows a particularly strong reaction.

been made by Hodneland *et al*. (1997) and Kocan *et al*. (1999, 2006).

The degree of cellular response of fish hosts to the infection is highly variable in different host species, and even within one host species. A light and electron microscopic study of the tissue response in three host species – plaice, haddock and rainbow trout – was undertaken by McVicar and McLay (1985). Infection in all three species elicited a chronic inflammatory response, which, depending on the duration of infection in the tissue and, to some extent, the host species, consisted variably of lymphocytes, macrophages, epithelioid cells, giant cells, fibrocytes and eosinophilic granular cells. The resulting granuloma was focal around individual *Ichthyophonus* bodies or obliterative around groups of parasitic bodies. Deposition of melanin has been noted to occur around foci of infection in some fish species such as herring (Sindermann and Scattergood, 1954), but not in all.

Changes in behaviour of hosts infected with *Ichthyophonus* have been noted, mostly associated with the debilitating effects of the infection. Most or all of the pathogenesis of *Ichthyophonus* can be linked directly to the replacement, disruption and atrophy of infected tissues by the proliferation of the parasite with, in extreme cases, the normal tissues of organs being replaced almost completely, leading to organ failure. Cardiac damage causing reduced swimming stamina in salmonids is a major feature associated with *Ichthyophonus* infection (Kocan *et al.*, 2006) and it is probable similar effects are commonly found in other species. The degree of directly

lytic activity associated with the nutrition of the parasite is uncertain in living fish, but this is evident in newly dead fish, as the germination tubes of the developing spores cause extensive destruction of surrounding tissues. As suggested by McVicar and McLay (1985), the success of the fish host in preventing invasion or in containing infection, once present, probably greatly influences the level of pathogenicity of *Ichthyophonus.* Aspects of the host's response have been implicated in the intra- and interspecific variations in fish susceptibility to *Ichthyophonus*. Experimental evidence for the important role of the cellular response in containing *Ichthyophonus* infections in the body of fish was obtained by Perry *et al*. (2004) by suppressing the cellular immune response by corticosteroid treatment of starry flounder (*P. stellatus)*. This resulted in a progression from latent to patent histologically identifiable *Ichthyophonus* infections.

Behavioural changes have also been reported by Sherman (2008) with newts chronically infected with an *Ichthyophonus*-like organism. These actively selected warmer temperatures than did healthy newts, as a consequence of the chronic nature of the infection.

Food-quality Aspects

The spoilage characteristics of fish flesh as food by *Ichthyophonus* have been well known for many years. Williamson (1913) used the terms 'spotted haddock', 'greasers' and 'smelly haddock' to describe haddock infected with *Ichthyophonus* caught off western Scotland. As pointed out by McVicar (1982), at least one of these terms has persisted in common usage in the fish processing industry to the present day, indicating the continual concern about this condition throughout that period. Sindermann (1958) reported that acute infections in herring caused degeneration and necrosis of the body muscles and that such fish were poor for smoking and pickling. The fillets of infected herring from both North American and European waters are characterized by being very soft and slimy, with strong off odours and often with pigment deposition around sites of infection. Spanggaard (1996) observed that products made from infected herring fillets were characterized by soft texture, unfavourable flavour changes and often had open holes from the necrotic tissue. Similar problems were reported by Kocan *et al*. (2004), who noted that *Ichthyophonus* infected Chinook salmon (*O. tshawytscha*) in the Yukon River smelled mildly 'fruity', did not dry properly when smoked and had white spots in the muscle and other tissues. To determine how the characteristic germination of the parasite after the death of its host contributed to increased spoilage, it is important to understand the factors influencing growth in fish products. Spanggaard (1996) and Spanggaard and Huss (1996) carried out detailed studies of the growth conditions of *Ichthyophonus* in North Sea herring products. Temperatures between 0 and 25°C and pH between 3 and 7 did not affect the growth ability of the parasite, but the significant effect of increasing concentrations of NaCl showed that it was unlikely that *Ichthyophonus* would develop and spoil processed products, such as pickled or salted herring, by continued growth. Both heating at 40°C and freezing at –20°C killed the parasite. The problems associated with processing infected fish are illustrated by the development of US patent 6821546 (Kimura *et al*., 2004), which outlines the use of a thiol protease inhibitor process for production of surimi from fish infected *I. hoferi* to deal with the associated very high protease activity in infected Alaskan pollock.

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20 Shellfish Diseases (Viral, Bacterial and Fungal)

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Introduction

Some of the earliest records of mass mortalities of shellfish were caused by microbial disease agents, e.g. the phycomycete fungus, *Ostracoblabe implexa*, responsible for 'Maladie du pied' in European oysters (*Ostrea edulis*) (Montauge, 1878) and the iridoviral agent of 'Maladie des branchies' in Portugese oysters (*Crassostrea angulata*) (Comps, 1988). Many excellent reviews and synopses cover the literature on microbial disease agents of shellfish (Lauckner, 1980, 1983; Sparks, 1985; Sindermann and Lightner, 1988; Brock and Lightner, 1990; Hanlon and Forsythe, 1990; Sindermann, 1990; Lightner *et al.*, 1992a; Messick and Sindermann, 1992; Shariff *et al.*, 1992; Couch and Fournie, 1993; Elston, 1993; Bower *et al.*, 1994a). However, increasing diversification and intensification of shellfish aquaculture continue to provide a seemingly inexhaustible reserve of new or emerging microbial diseases and related research, diagnostic and management challenges. Advances in molecular techniques over the past 20–30 years have particularly broadened the scope for microbial pathogen research. This chapter updates the 1999 overview of microbial diseases of shellfish and methods applied to their diagnosis, control and treatment. Apparently non-significant pathogens have not been updated, except where they have transformed into serious disease agents (e.g. *Herpes* virus of abalone) or been removed, since, as in the 1999 review, the ability to distinguish between primary and opportunistic pathogens remains an essential factor for effective control or treatment.

Although biomolecular investigations of diseases have enhanced research investigations significantly, many viruses and bacteria continue to evade accurate identification, appearing in the midst of a disease investigation and disappearing thereafter. Molecular tools are also highlighting the need for constant revision of viral systematics. This chapter describes several new genera and families for viruses that have emerged from shrimp disease investigations alone. Molecular tools have also proven useful in grouping related microbial agents, despite very different species and geographic locations of infections. This is valuable for enhanced diagnostic/screening applications and effective disease control measures.

Notwithstanding the advances made with, and the value of, molecular detection and phylogenetic technology, the ongoing lack of robust cell lines for isolation of shellfish microbial disease agents continues to constrain understanding of their pathobiology and host–pathogen interface. Accurate

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interpretation of molecular assay results also remains a challenge in the absence of clinical disease or observation of the pathogen itself. This challenge is being addressed by more stringent 'non-gold standard' validation exercises; e.g. ISO 17025[1] and the World Organization for Animal Health (OIE)[2] validation processes for diagnostic kits applied to OIE listed pathogens (terrestrial and aquatic). However, few tests to date have passed these rigorous quality assurance/quality control validation procedures.

As detection and diagnostic technologies advance, microbial pathogens undoubtedly will remain many steps ahead of us, maintaining the ongoing challenge to researchers, managers and regulators. Of particular note for work over the next decade will be how shellfish – predominantly limited to coastal, brackish and freshwater environments – and their microbial fauna adapt to environmental changes brought about by climate change, intensification of human use of coastal waters and globalization of trade (ballast and animal transfers).

Viral Infections

Viruses of Bivalvia Linné, 1758

Iridovirus-like viruses

The first viral disease recorded from bivalves was 'Maladie des branchies' (gill disease), which appeared in Portuguese oysters (*C. angulata*) in France in 1966 (Marteil, 1969). The disease decimated Atlantic populations of Portugese oysters until they reached non-exploitable levels in the early 1970s.

Gills of infected oysters showed intense haemocyte infiltration, abscessing and necrosis. *Iridovirus*-like viral particles, named 'gill necrosis virus' (GNV) (Comps and Duthoit, 1976; Comps, 1988), were observed using transmission electron microscopy (TEM) in dense inclusion bodies in the cytoplasm of hypertrophied connective tissue cells and, to a lesser extent, in infiltrating haemocytes. Pacific oysters (*C. gigas*) imported from Japan and Pacific North America (Andrews, 1980) to replace affected Portuguese oyster populations also showed signs of infection, but their gill lesions regressed with negligible mortality (Comps, 1988).

GNV particles are non-enveloped, 350–380 nm in diameter, icosahedral, with two trilaminar layers enclosing an electron-dense core (250 nm in diameter) (Comps, 1988). GNV is cytoplasmic, Feulgen positive (indicative of a deoxyribovirus) but larger than Iridoviridae from other hosts (120–300 nm) (Francki *et al.*, 1991; Singleton and Sainsbury, 1991).

A similar *Iridovirus*-like agent, detected in the gills of Pacific oysters that had been imported into France from Japan, Korea and Canada between 1966 and 1969, was also reported, but not compared with GNV (Comps, 1970, 1972; Comps and Duthoit, 1979), and no similar agent has been reported from Pacific oyster gills since. GNV is believed to persist in relic populations of Portuguese oysters (Comps and Masso, 1978) in the Mediterranean, but there have been no futher reports of detections.

Mass mortalities of Portuguese oysters reported from a depuration plant, and in the Bay of Arcachon, France, between 1970 and 1973, were linked to an *Iridovirus*-like infection of the haemocytes (Comps *et al.*, 1976). Described as 'haemocyte infection virus' (with the unfortunate acronym, HIV), the infection was associated with discolouration of the digestive gland, disruption of connective tissue and haemocyte infiltration. Comps and Bonami (1977) noted an identical virus in the haemocytes of Pacific oyster from the Bay of Arcachon, but no detections of iridoviral-like haemocyte infections have been reported from either Portugese or Pacific oysters since.

Leibovitz *et al.* (1978) attributed significant mortalities of Pacific oyster larvae at two shellfish hatcheries in Washington State, USA, to another *Iridovirus*-like infection (Elston, 1979, 1980a; Elston and Wilkinson, 1985). The virus affected larvae over 150 µm in length and caused lesions in the velum and other ciliated epithelia, hence the name 'oyster velar virus disease' (OVVD). Mortalities of up to 100% typically occurred in

April and May, although occasional late summer mortalities were also reported (Elston and Wilkinson, 1985). Surviving oysters showed no evidence of infection but were suspected of being reservoirs of OVVD (Elston, 1993). Feulgen-positive, cytoplasmic inclusion bodies were comprised of virogenic stromae that produced bicapsid icosahedral virions, 228 nm in diameter (Elston and Wilkinson, 1985).

Birnavirus-like viruses

A double-stranded RNA virus, similar to infectious pancreatic necrosis virus (IPNV) of salmonids, was isolated from the digestive gland of diseased tellin clams (*Tellina tenuis*) from Scotland (Buchanan, 1973; Hill, 1976a) on bluegill fibroblast cell lines (Hill, 1976a,b; Hill and Alderman, 1979). *Tellina*-virus (TV) was designated as a member of the Birnaviridae (bisegmented, double-stranded RNA viruses) (Dobos *et al.*, 1979); however, the exact taxonomic status for TV has never been established. This is also the case for similar viruses isolated from Pacific oysters, American oysters (*C. virginica*) and European oysters (*O. edulis*), hard-shell clam (*Mercenaria mercenaria*) and two gastropods, common periwinkle (*Littorina littorea*) and keyhole limpet (*Patella vulgata*), using the same cell line (Hill and Alderman, 1979). Experimental infections of oysters using preparations from the cell-line isolates caused extensive haemocyte infiltration and necrosis of the digestive gland. Infected cells contained paracrystalline arrays of icosahedral particles, 66 nm in diameter, which were similar, but not identical, to the intracytoplasmic phages originally described from tellin clams (Buchanan, 1978). Hill *et al.* (1986) concluded that the virus might have been a cell-line contaminant, rather than a primary shellfish pathogen.

Other investigators have isolated viruses from shellfish using non-shellfish cell lines. Lo *et al.* (1988) isolated a hexagonal IPN-like virus (62 nm diameter) from discoloured gills of the clam, *Meretrix lusoria*, using an insect cell line, and Meyers (1979a) isolated a reo-like virus (79 nm diameter) from American oysters using a fish cell line. The significance of these viruses to the shellfish has not been pursued as no diseases attributable to these isolates have been reported since.

Herpes-like viruses

The first *Herpes*-like infection was reported by Farley *et al.* (1972) from the haemocytes of moribund American oysters held in thermal effluent (28–30°C) from a power plant in Maine, USA. Mortalities of 52% were attributed to Feulgen-positive inclusions in the haemocyte nuclei of moribund specimens (Fig. 20.1). Similar inclusions were found in wild, apparently healthy, oysters at the same site. TEM investigation revealed the presence of non-enveloped virions, 70–90 nm in diameter, in the nuclei of infected cells. Enveloped particles, 200–250 nm in diameter, occurred in the cytoplasm. Cowdry type A intranuclear inclusion bodies (Cowdry, 1934)[3] and morphological and nucleic acid characteristics resembled those of other *Herpes*-type viruses (Farley, 1978; Francki *et al.*, 1991).

A *Herpes*-like virus was also found in 7- to 11-day-old hatchery-reared Pacific oysters in New Zealand (Hine *et al.*, 1992). Hypertrophied haemocyte nuclei contained non-enveloped virions, 97 ± 4 nm in diameter. Enveloped and tegumented (capsid-covering characteristic of the Herpesviridae, which may or may not be enclosed in an envelope) viral particles, 131 ± 9 nm in diameter, were found in cytoplasmic vacuoles and extracellularly. Nicolas *et al.* (1992a) and Le Deuff *et al.* (1994) found *Herpes*-like infections in hatchery-reared Pacific oyster larvae in France and similar infections were detected in 3- to 7-month-old Pacific oysters on open-water oyster beds (Renault *et al.*, 1994). As with the other *Herpes*-like infections, mortalities were most severe during warmwater periods and appeared to be linked to overcrowding or other stress-related factors (Buchanan and Richards, 1982). Filtered homogenates of infected larvae were inoculated into cultures of Pacific oyster larvae and reproduced the mortalities observed under hatchery conditions (Le Deuff *et al.*, 1994); however, similar experiments with European oyster

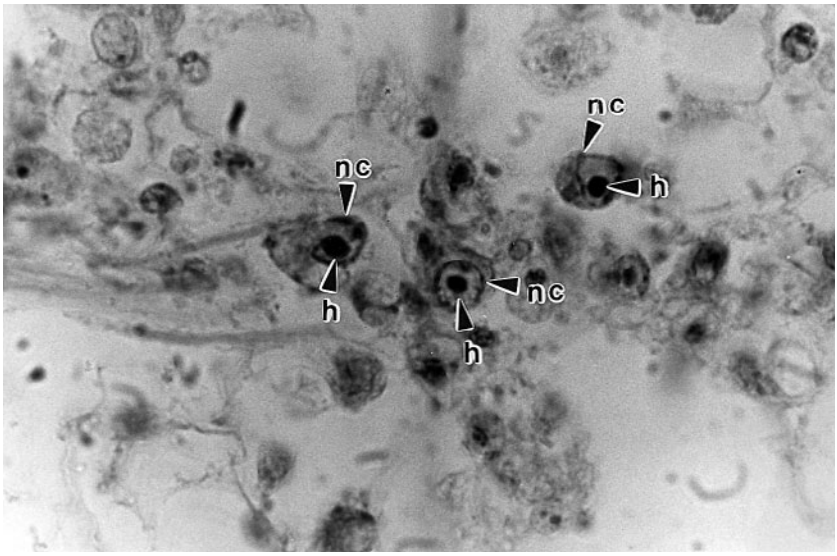


Fig. 20.1. Intranuclear *Herpes* sp. infection of *Crassostrea virginica* haemocytes (h, *Herpes* inclusion; nc, nuclear chromatin). Original histology slide provided by C.A. Farley. (H & E, ×1000).

larvae were inconclusive. The virions in Pacific oyster resemble those described by Hine *et al.* (1992), measuring approximately 80 nm in diameter; however, both non-enveloped and enveloped stages were detected in the cytoplasm. Enveloped virions, 125 nm in diameter (size calculated from Renault *et al.*, 1994), have spike-like protrusions on the surface, a feature characteristic of Herpesviridae (Francki *et al.*, 1991). In contrast to other *Herpes*-like infections described in bivalves, however, no haemocyte infiltration was observed in the hatchery-reared Pacific oyster (Le Deuff *et al.*, 1994; Renault *et al.*, 1994). It is possible that these infections are related to an earlier record by Alderman (1980), who suggested a viral aetiology for 'summer mortality' in Pacific oyster from North Wales based on his observation of Cowdry type A inclusions in tissues examined from moribund oysters.

Comps and Cochennec (1993) reported another *Herpes*-like virus observation in 5-month-old European oysters suffering 90% mortality on oyster beds in north-eastern France. Infected haemocytes contained Feulgen-positive, intranuclear inclusions comprised of spherical or polygonal particles 80 nm in diameter. Enveloped paraspherical virions, 160–180 nm in diameter, occurred in the cytoplasm. As with infections of Pacific oyster, mortalities in European oyster juveniles coincided with warm summer water temperatures.

Subsequent studies have revealed this virus in several larval and juvenile bivalve species in Europe, including Pacific oyster, carpet clam (*Ruditapes decussatus*), Manila clam (*R. philippinarum*) and Coquille St Jacques scallop (*Pecten maximus*) (Renault *et al.*, 2000, 2001; Renault and Arzul, 2001; Renault and Novoa, 2004), and the virus has been assigned the taxonomic designation OsHV-1 for Ostreid Herpesvirus-1 (Arzul *et al.*, 2001a,b,c; Renault *et al.*, 2001; Davison *et al.*, 2005). The virus appears to induce apoptosis, characterized by chromatin condensation into dense crescent-shaped aggregates lining the nuclear membrane, followed by nuclear collapse. Nuclei of infected cells contained circular to polygonal empty capsids (82±4 nm in diameter) and nucleocapsids (74±4 nm in diameter). Extracellular viruses were usually enveloped (111±5 nm in diameter). A tail was rarely observed. It has been suggested that

these detections may be related to the introduction of non-native species for aquaculture (Arzul and Renault, 2002; ICES, 2004; IFREMER, 2009), with evidence based on the presence of viral DNA and protein in subclinical specimens (Arzul *et al.*, 2002; Barbosa *et al.*, 2004). Recently, the same infection was identified as the cause of abnormally high (40–100%) mortalities of Pacific oyster throughout most of France.[4] Environmental conditions conducive to early gametogenesis were related to decreased immunocompetence against infection by the OsHV-1 virus, as well as other opportunistic pathogens, e.g. *Vibrio splendidus* (Gagnaire *et al.*, 2006, 2007; Samain *et al.*, 2007). The level of losses posed a significant threat to seed production in Europe. The same pathogen has been associated with hatchery losses of Pacific oyster in California (Burge *et al.*, 2007) and New Zealand (Webb *et al.*, 2007), which highlights the ubiquitous distribution of the virus and the potential for significant losses if environmental conditions elsewhere compromise oyster immunocompetence.

Gonadal tumour cells in hard-shell clams (*M. mercenaria*) from Rhode Island have also been associated with nuclear inclusion bodies and peripheral displacement of host chromatin (Barry and Yevich, 1972). Although described by Farley (1978) and Sindermann (1990) as *Herpes*-like inclusion bodies, ultrastructural investigations by Hesselman *et al.* (1988) showed a 'haloed granular substance' with no evidence of viral particles. The same authors studied clam populations (and possibly species) different from those examined by Barry and Yevich (1972); however, it appears likely that Cowdry-type nuclear inclusions may have more than one cause and no subsequent detection of viral particles from bivalve gonadal tumours have been reported since.

Papova-like viruses

Farley (1976a, 1985) described a lytic virus in hypertrophied gonadal cells of American oysters from the north-eastern USA (Fig. 20.2a,b). Similar infections were found in the same species from Atlantic Canada (McGladdery and Stephenson, 1994) and the Mexican (Aguirre-Macedo *et al.*, 2007) and Floridian (Winstead and Courtney, 2003; Winstead *et al.*, 2004) coasts of the Gulf of Mexico. Similar/identical infections have also been described from Pacific oyster in Europe (Garcia *et al.*, 2006; Watermann *et al.*, 2008) and Korea (Choi *et al.*, 2004). The infection, described as viral gametocytic hypertrophy (VGH) or ovocystis, is caused by a *Papilloma*-like virus (family Papovaviridae), with icosahedral morphology, non-enveloped capsids (50–55 nm in diameter) (Fig. 20.3a–c) and Feulgen-positive histochemistry (Farley, 1976b, 1978, 1985). Viral replication is intranuclear and, rather than infecting the gametes, elicits massive hypertrophy in the germinal epithelium. Infection levels of VGH are generally low (fewer than five infected cells per 6 µm tissue section); however, up to 80% prevalence and 37 lesions/tissue section occur sporadically (McGladdery and Stephenson, 1994). Although the massive hypertrophy and tissue displacement surrounding infected cells is significant, no adverse effect on oyster health or fecundity has yet been documented (Garcia *et al.*, 2006).

A papova-like virus has also been reported from gold-lipped pearl oysters (*Pinctada maxima*) from northern Australia (Humphrey *et al.*, 1999). Infections were observed in the labial palps of two pearl oysters during a routine histological examination of samples fished from the western Torres Strait region. The infections were characterized by massive hypertrophy of the epithelial cells and nuclei of the ciliated columnar epithelium of the inner surfaces of the labial palps. Affected nuclei were up to seven times larger in diameter than those of unaffected epithelial cells. The nuclear chromatin was marginated and affected nuclei contained a Feulgen-positive central inclusion. In many cases, this was separated from the peripheral nuclear chromatin by a clear zone. Under electron microscopy, non-enveloped icosahedral virus-like particles (about 60 nm in diameter) were observed in the central mass of hypertrophied nuclei. The authors could not

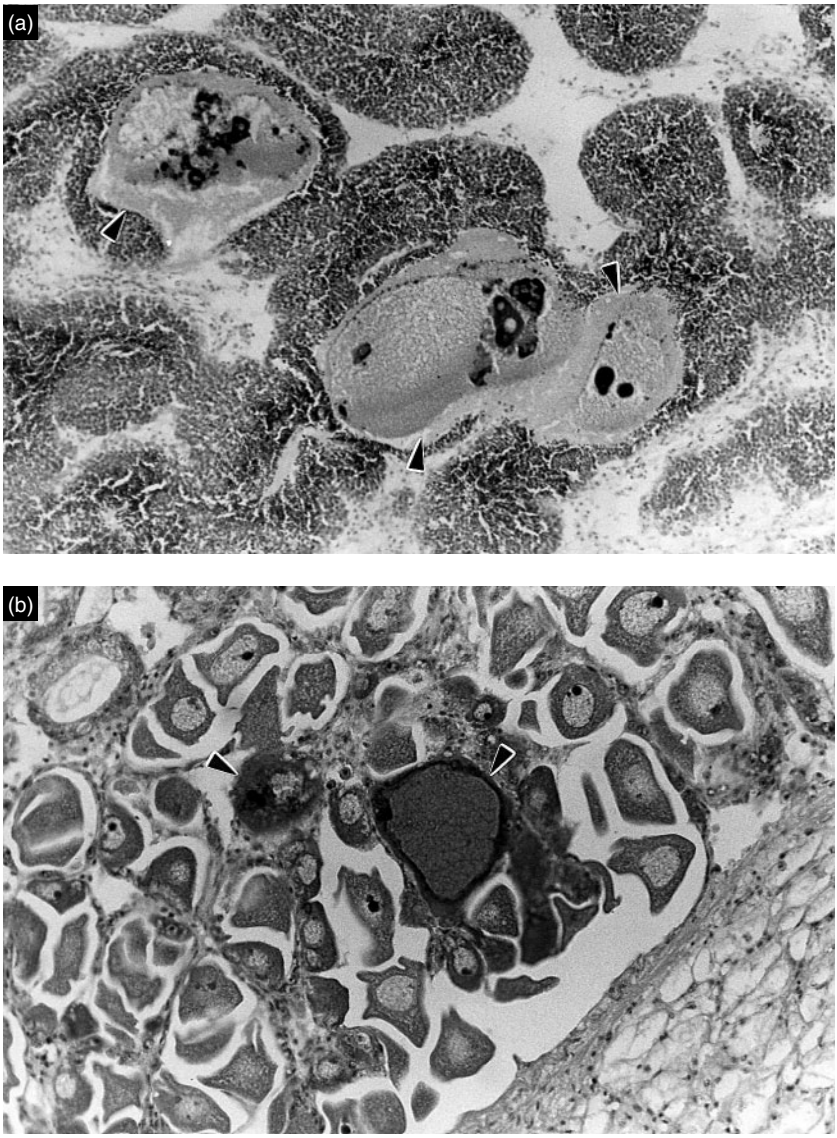


Fig. 20.2. (a) Viral gametocytic hypertrophy (VGH) *Papovavirus*-like infection of the gonad of a male *Crassostrea virginica* (arrows = hypertrophied cells) (H & E, ×160). (b) VGH infection of the gonad of a female *Crassostrea virginica* (arrows = hypertrophied cells) (H & E, ×160).

determine if the infections impacted the health of wild populations due to the low prevalence of detection (4%); however, as pearl oyster culture intensifies, monitoring for the presence of such viruses may be warranted.

Retro-like viruses

The first successful isolation of a non-vertebrate reovirus and experimental reproduction of bivalve infection were accomplished by Oprandy *et al.* (1981)

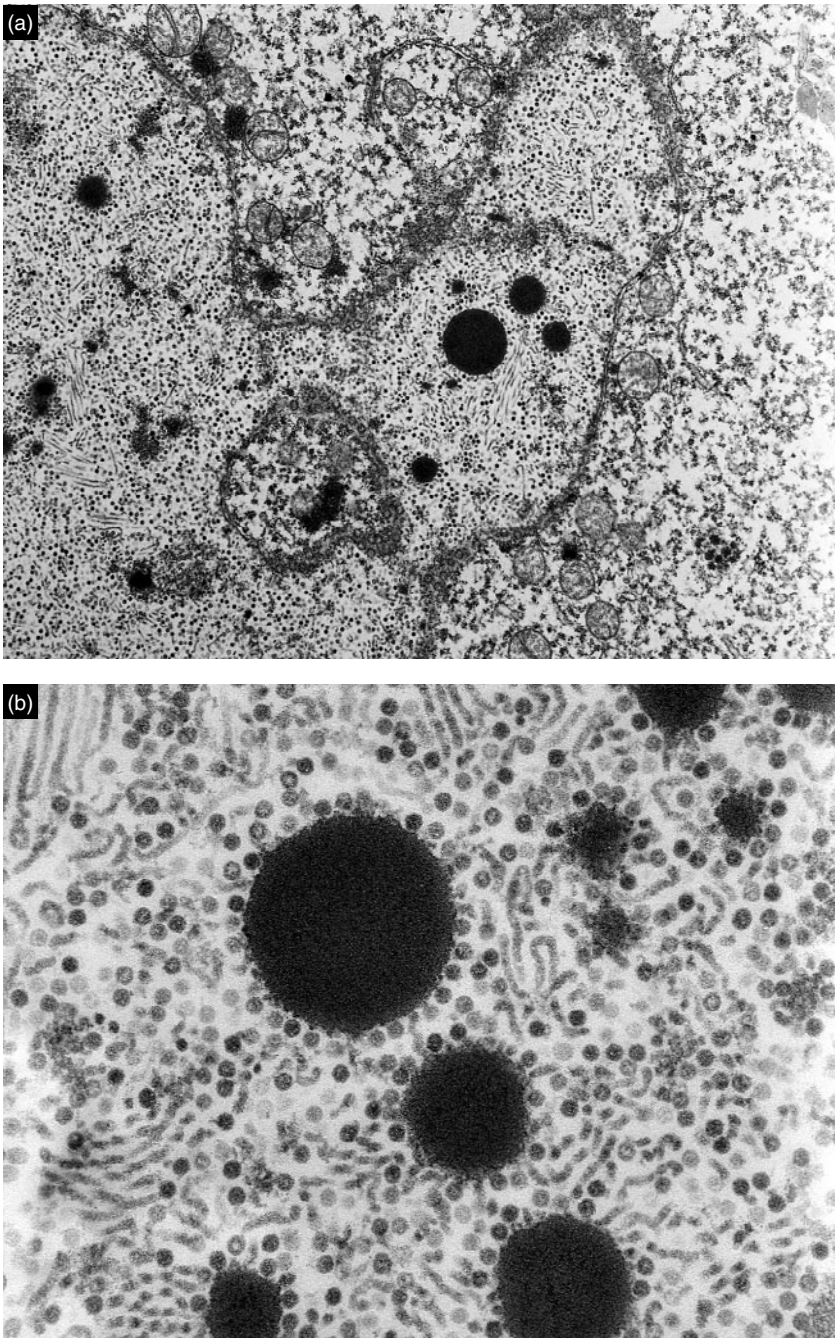


Fig. 20.3. (a) VGH infection of the nucleus of a germinal cell of *Crassostrea virginica*. Note distension of the nuclear membrane and aggregation of mitochondria in the surrounding cytoplasm (TEM, ×3000). (b) VGH virogenesis in a germinal cell of *Crassostrea virginica*. Note virions budding off electron dense viral stromae (VS) and filamentous forms (TEM, ×11,000).

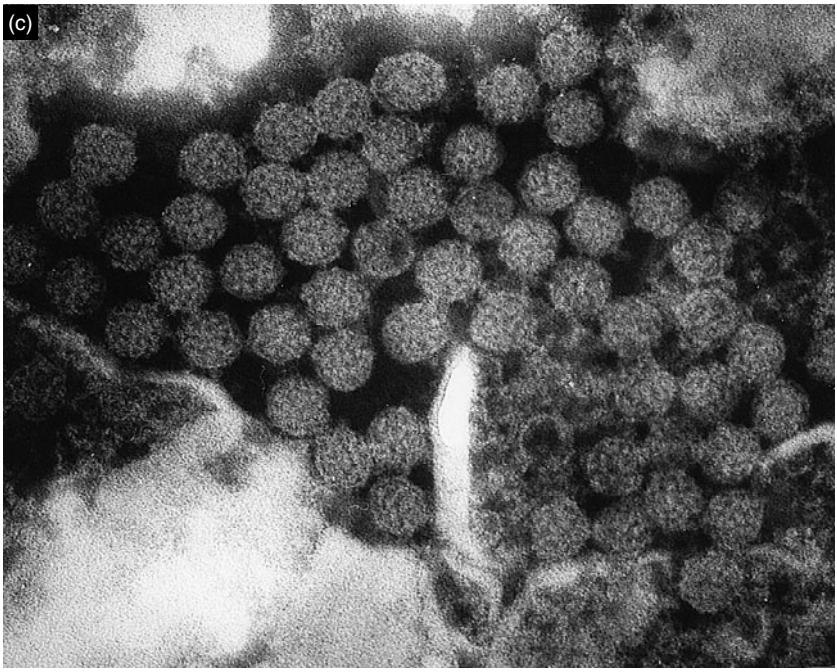


Fig. 20.3. (c) VGH virions showing icosahedral and subunit morphology. Photograph courtesy of J.-C. Veyrunes and M. Bergoin (negative stain, TEM, ×63,000).

with a *Retro*-like virus from soft-shell clams (*Mya arenaria*) from the north-eastern USA affected by haemic neoplasia. The virus measured 120 nm in diameter, had ultraviolet (UV) absorbance and density characteristics consistent with those of mammalian retroviruses (Francki *et al.*, 1991). Oprandy and Chang (1983) enhanced viral replication and neoplasia using 5-bromodeoxyuridine (5-BrdUrd) (which induces retrovirus expression in mammalian cell culture). Noël (1992) used the same technique to induce *Retrovirus*-like particles detected in a small number of bay mussels (*Mytilus trossulus*) also affected by haemic neoplasia. Although the number of particles was low, compared with the level of neoplasia, the virus was detected in direct association with the neoplastic nuclei (Fig. 20.4). Retroviral involvement in disseminated neoplasias is also reported from edible cockles (*Cerastoderma edulis*) from Spain (Romalde *et al.*, 2006). Cause and effect, however, remain to be determined due to the complexity of environmental factors that may also play a direct or predisposing role.

Picorna-like viruses

Granulocytomas in 4% of blue mussels (*M. edulis*) (n=900) from Denmark were associated with a non-enveloped, icosahedral, Feulgen-negative virus, 27 nm in diameter (Rasmussen, 1986). These characteristics are shared by the Picornaviridae (Singleton and Sainsbury, 1991). In contrast, Elston *et al.* (1992) found similar particles in ultramicrographs of *Mytilus* species from North America with haemic neoplasia. These were interpreted initally as the swollen nuclear pores of neoplastic nuclei; however, since early developmental stages of neoplasia can resemble granulomas (Elston *et al.*, 1992), possible *Picornavirus* involvement cannot be ruled out (Elston, 1997).

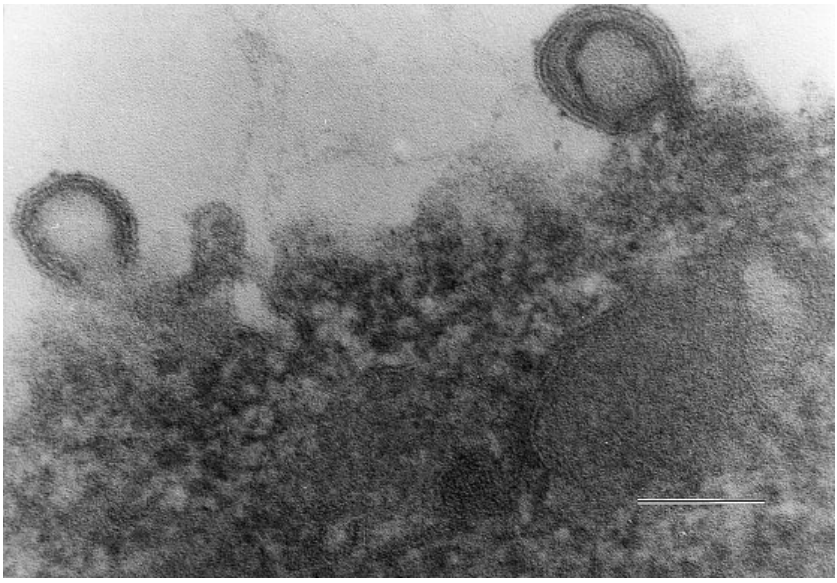


Fig. 20.4. *Retrovirus*-like virions associated with a neoplastic haemocyte of the mussel, *Mytilus trossulus*. Photograph courtesy of D. Noël (scale bar, 90 nm).

Virus-like particles were also observed in green lipped mussel (*Perna canaliculus*) spat (15–30 mm in shell length) suffering high levels (50–100%) of mortality following thinning and reseeding of culture lines in Marlborough Sound, New Zealand, in early 1994 (Jones *et al.*, 1996). Mortalities (2–5%) of adult mussels (75–110 mm in length) at the same locations as in 1994 were also associated with the virus-like particles. Similar granulomatous cell pathology and particles observed in stunted gallo mussels (*M. galloprovincialis*) in the same area were speculated to have been related to heavy mortalities of mussel spat in the same areas in the 1980s; however, no evidence of a direct correlation has been reported since.

Granulocytomas are common in mussels and, in some areas, are associated with environmental pollution (e.g. Lowe and Moore, 1979; in the UK) or soft tissue irritation from sand, grit and/or zooplankton incursions (e.g. McGladdery *et al.*, 1993) without evident stunting or other clinical signs. Possible viral involvement, therefore, is rarely investigated.

Reoviruses

Meyers (1979a) and Meyers and Hirai (1980) were the first to describe a putative fish reovirus from American oysters, designated reovirus $13p_2$. Infection trials showed apparent pathogenicity to finfish (bluegill and rainbow trout), triggering analysis of the potential for bivalves to act as reservoirs of finfish viral infections (Meyers, 1984). No further work has been undertaken on fish reoviruses since, although similar studies on other fish viruses and the role of bivalves as reservoirs or carriers have been pursued (e.g. Mortensen *et al.*, 1998). Regardless of microbial entity, this area of microbial research will likely gain more attention as 'integrated multitrophic aquaculture' (IMTA) develops and gains greater economic significance (e.g. Lander *et al.*, 2004).

Viruses of uncertain affinity

An unidentified virus was detected by Pass *et al.* (1988) during investigation of mass mortalities of the gold-lipped pearl oyster in

Australian waters. Viral-like stromae containing presumptive virions (approximately 100–105 nm in diameter) were found in the nuclei of hypertrophied digestive tubule epithelia (Perkins, 1993); however, further investigation led to the conclusion that the mortalities were due to a bacterial syndrome associated with transportation stress (see the section below on Gram-negative bacteria of bivalves) rather than to the presence of the virus (Pass *et al.*, 1987).

Another unidentified viral infection was reported from an epithelial cell in the digestive gland of a single, healthy, Japanese scallop (*Patinopecten yessoensis*) examined as part of a histological study of nutrient accumulation (Chang *et al.*, 2002). The infected cell showed several layers of roughly parallel, enveloped, rod-shaped virions averaging 520×130 nm, but no occlusion bodies. The only other abnormality noted in the infected cell was a large number of small (520–1050 nm in diameter), double-membrane vacuoles that appeared to be a network of tubular smooth endoplasmic reticulum among the viral particles. No other reports of such an infection have been noted since the description of Chang *et al.* (2002).

Viruses of Gastropoda Cuvier, 1797

Herpes-like viruses

A *Herpes*-like virus associated with 'crack-shell disease' and a ganglioneuritis condition of Japanese disc abalone (*Haliotis discus hannai*) and tokobushi/'variously coloured'/'round' abalone (*H. diversicolor*) was reported from various culture facilities in China and Australia. Since its emergence in 1999, abalone farms in southern China have suffered heavy losses of all sizes of abalone associated with digestive tract, hepatopancreas, renal tissue, haemocyte and neural tissue pathology (Song *et al.*, 2000; Nie and Wang, 2004; Wang *et al.*, 2004). Temperatures less than 20°C appear to trigger clinical expression of the disease (Wang *et al.*, 2004) and, in Taiwan, the nervous system was reported to be the primary target tissue (Chang *et al.*, 2005). A wide range of abalone species are affected by the virus: *H. discus hannai*, *H. diversicolor* (including *H. diversicolor aquatilis*, *H. diversicolor supertexta* and *H. diversicolor diversicolor*), green-lip abalone (*H. laevigata*) and black-lip abalone (*H. rubra*), which was proposed as a *Herpes*-like virus by Chang *et al.* (2005) based on size and icosahedral morphology. Similar infections were detected in blue mussels in the same area of Japan as diseased abalone and artificial infections were established in the turban shell (*Turbo* sp.) and tegula (*Tegula* (=*Chlorostoma*) *rusticum* (Li *et al.*, 2000), suggesting wide host specificity.

In all, four types of viral-like particles have been reported from two Australian abalone species (Zhengli and Handlinger, 2004); thus, the precise relationship of specific *Herpes*-like particles to the ganglioneuritis disease remains unclear. *V. alginolyticus* and *V. parahaemolyticus* have also been reported from infected tokibushi abalone and may be co-factors in the disease (Wang *et al.*, 1999; Zhang *et al.*, 2001a,b). An epizootic fatal wasting disease was associated tentatively with retroviral-like particles in discus abalone from western Japan (Otsu and Sasaki, 1997; Nakatsugawa *et al.*, 1999), and was also associated with signs of glioma or tumour-like lesions (Nakatsugawa *et al.*, 1988; Harada *et al.*, 1993). The cause–effect relationship between these viral infections and the concomitant lesions and mortalities, as well as environmental factors, however, remains unclear.

Despite this complex and unresolved aetiology, 'abalone *Herpes*-like disease' was listed by the World Organization for Animal Health (OIE) in 2009 as a disease that required attention with respect to trade movements and health certification of abalone and certain abalone products (www.oie.int – see OIE Aquatic Code 2009a,b). Specific disease information 'fact sheets' for screening and diagnosis are in preparation to assist countries to implement the measures outlined in the Code pending inclusion of a chapter in the OIE *Manual*

of Diagnostic Tests for Aquatic Animals (OIE, 2009a).

Viruses of Cephalopoda Cuvier, 1797

Devauchelle and Vago (1971) were first to report a virus from a cephalopod host. Non-enveloped particles, 75 nm in diameter, were found in the epithelial cells of the stomach of a cuttlefish (*Sepia officinalis*), forming intracytoplasmic microtubular arrays. Mature particles formed paracrystalline arrays. The authors suggested a *Reovirus*-like identification, based on the ultrastructural characteristics of the virus; however, no pathogenic effects from the infection were observed. The only other virus reported from a cephalopod was one found in sections of necrotic muscle tissue in common octopus (*Octopus vulgaris*) from the Bay of Naples, Italy (Rungger *et al.*, 1971). The hexagonal particles, 120–140 nm in diameter, were identified tentatively by Farley (1978) as Iridoviridae; however, this has yet to be confirmed (Hanlon and Forsythe, 1990).

Viruses of Decapoda Latreille, 1803

Nimaviridae

A recent complex of viruses has emerged that has had a devastating impact on shrimp production throughout most shrimp producing countries. The complex is named after the grossly visible 'white spots' in the epicuticle, which are caused by abnormal deposition of calcium salts by the cuticular epidermis. The complex encompasses a range of diseases originally described as:

- Baculoviral hypodermal and haematopoietic necrosis virus (BHHNV),[5] shrimp explosive epidermal disease (SEED), China virus disease; all reported from China;
- Rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ), reported from Japan, China and Korea;
- Systemic ectodermal and mesodermal baculovirus (SEMBV), red disease, white spot disease; all reported from Thailand; and
- White spot baculovirus (WSBV), white spot syndrome virus (WSSV) and white spot disease; reported from Indonesia, Taiwan, Vietnam, Malaysia, India and Texas, USA.

In 2000, the OIE listed WSD as a reportable disease due to its economic severity, rapid spread throughout the Asia–Pacific region in a wide range of shrimp species and its apparent very low host specificity. Since 2000, intense molecular investigations have revealed that all these infections appear attributable to a complex known as white spot syndrome virus (WSSV). This is designated by the International Committee on Taxonomy of Viruses (ICTV) as a single species, encompassing a range of geographically and genotypically distinct variants. WSSV-1 is assigned as the first (and currently sole) species in a new genus, *Whispovirus*, within a new family, Nimaviridae (Vlak *et al.*, 2004).

WSD viral particles are ovoid or ellipsoid/bacilliform measuring 120–150 × 270–290 nm and are characterized by a distinct thread- or flagellum-like apical extension (Vlak *et al.*, 2004).

WSD has an extremely wide host range within the decapoda. *P. monodon*, *P. japonicus*, *P. chinensis* (=*orientalis*), *P. indicus*, *P. merguiensis*, *P. penicillatus* and *P. setiferus* are all susceptible to infection, and experimental infections have been produced in *P. vannamei*, *P. stylirostris*, *P. aztecus*, *P. duorarum* and *P. setiferus*, suggesting no apparent resistance by any penaeid species (Lightner, 1996a). Many non-penaeid crustaceans from marine, estuarine and freshwater environments have also been found to carry evidence of WSSV-1, including species from all three families of freshwater crayfish (Corbel *et al.*, 2001; Jiravanichpaisal *et al.*, 2001; Edgerton *et al.*, 2004). This has led the OIE to list all decapod crustaceans as potential carriers of WSSV (OIE, 2009a), the first Order level designation for susceptible

species applied to an OIE aquatic animal listed disease.

Viruses of Penaeidae Boas, 1880

Baculoviridae

The first baculovirus described from shrimp was *Baculovirus penaei* Couch (BP) in pink shrimp (*P. duorarum*) from Florida (Couch, 1974a,b). Bonami, Bruce, Poulos and Lightner (unpublished, cited in Bruce *et al.*, 1993) proposed changing BP to 'PvSNPV' ('the most characterized variant, from *P. vannamei* [white-leg shrimp], in the singly enveloped nuclear polyhedrosis virus group') in compliance with the guidelines for virus nomenclature designated by the ICTV. For the sake of consistency with previous descriptions, however, the acronym 'BP' is used here.

BP is reported from 12 species of *Penaeus* (Lightner *et al.*, 1992b), including brown shrimp (*P. aztecus*, *P. subtilis* and *P. californiensis*), white-leg shrimp (*P. vannamei*), pink shrimp (*P. duorarum*), roughback shrimp (*Trachypenaeus similis*), white shrimp (*P. setiferus* and *P. schmitti*), Pacific blue shrimp (*P. stylirostris*), red-tail shrimp (*P. penicillatus*), red-spotted shrimp (*P. brasiliensis*) and red shrimp (*P. paulensis* and *P. marginatus*), ranging from the Gulf of Mexico, south to Brazil and in the Pacific from Peru to Hawaii. Of these species, white-leg shrimp, brown shrimp, pink shrimp and red shrimp are affected most severely, with serious mortalities in commercial hatcheries at larval, postlarval and early juvenile stages. Occlusion bodies have also been found in up to 80% of wild pink and brown shrimp; however, no mass mortalities in wild populations have been attributed to BP (Couch, 1978; Brock and Lightner, 1990).

Clinical signs of BP infections are nonspecific: gill and surface fouling, decreased growth and anorexia. Severe infections result in necrosis and loss of hepatopancreatic and midgut epithelia. Transmission occurs at all life stages, but patent infections (detectable occlusion bodies) may not develop in older shrimps. Pyramid-shaped occlusion bodies occur principally in the nuclei of the hepatopancreas and midgut epithelia (Fig. 20.5a) and are released in the faeces. Up to six occlusion bodies, measuring 0.5–20 µm across the base, can be found in a single nucleus and may contain rod-shaped virions measuring 55–75×300 nm (Fig. 20.5b) (Couch, 1974a).

A second baculoviral-like infection of shrimps is found in cultured shrimp throughout Asia, the Middle East, Italy, South America, the southern USA and India (Lightner and Redman, 1981; Lightner *et al.*, 1992b; Nativiidad and Lightner, 1992; Ramasamy *et al.*, 1995). Although originally described as '*P. monodon*-type' baculovirus (MBV), infections have since been detected in a wide range of penaeid species (Lightner and Redman, 1992), and a similar baculovirus in eastern king prawn (*P. plebejus*) in Australia ('plebejus baculovirus' – PBV) (Lester *et al.*, 1987) is considered to be a geographic variant of the MBV-type virus (Doubrovsky *et al.*, 1988; Lightner *et al.*, 1992b). In addition to giant tiger prawn, MBV infections have been detected in banana prawn (*P. merguiensis*), green tiger prawn (*P. semisulcatus*), grooved shrimp (*P. kerathurus*), white-leg shrimp, brown tiger shrimp (*P. esculentus*), red-tail shrimp, eastern king prawn and greasyback/sand shrimp (*Metapenaeus ensis*). The diverse host and geographic range of MBV-type baculoviruses strongly suggests a group of morphologically related strains, rather than a single species. Mari *et al.* (1993) proposed renaming MBV as PmSNPV ('the most characterized variant, from *P. monodon*, in the singly enveloped nuclear polyhedrosis virus group'), which is now considered the taxonomic designation; however, for the sake of consistency, MBV is used here.

MBV is most pathogenic to giant tiger prawn larvae, with lesser effects in banana prawn and green tiger prawn larvae (Johnson and Lightner, 1988); however, intense infections may also occur in apparently healthy shrimp. Determination of MBV pathogenicity is further complicated by multi-aetiology infections and senility (Lightner *et al.*, 1992b). Clinical signs include lethargy, anorexia, stunted growth, dark discoloration

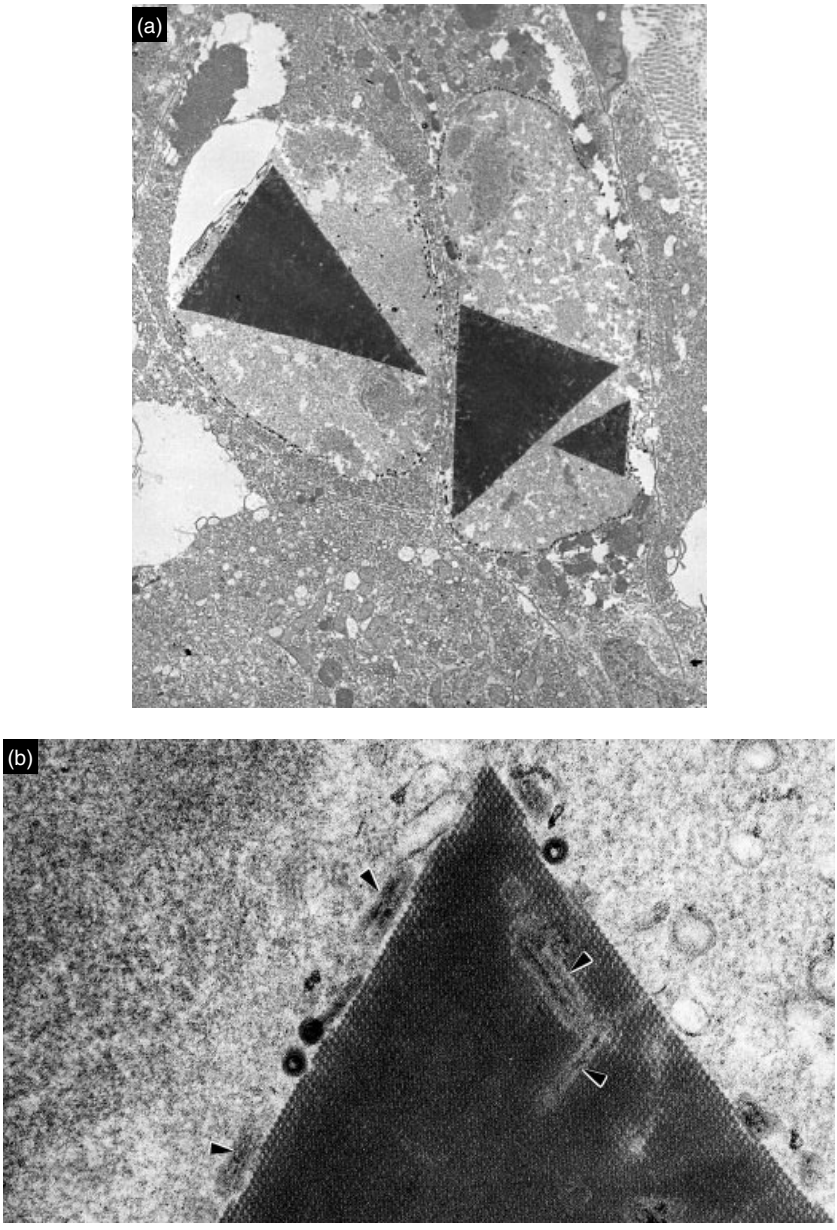


Fig. 20.5. (a) *Baculovirus penaei* (BP) occlusion bodies in the nucleus of hypertrophied hepatopancreas cells of *Penaeus aztecus*. Photograph courtesy of D.V. Lightner (TEM, ×5400). (b) Occlusion body of BP containing rod-shaped virions (arrows). Note virions also lying around the edge of the occlusion body. Photograph courtesy of D.V. Lightner (TEM, ×61,200).

and surface fouling, such as *Leucothrix mucor* (bacteria) or *Zoothamnium* spp. (ciliates). Acute MBV infections cause loss of hepatopancreas and midgut epithelia, although this may also be associated with secondary *Vibrio* infections (Lightner *et al.*, 1983a). Adult and juvenile stages appear more resistant than larvae, and stress

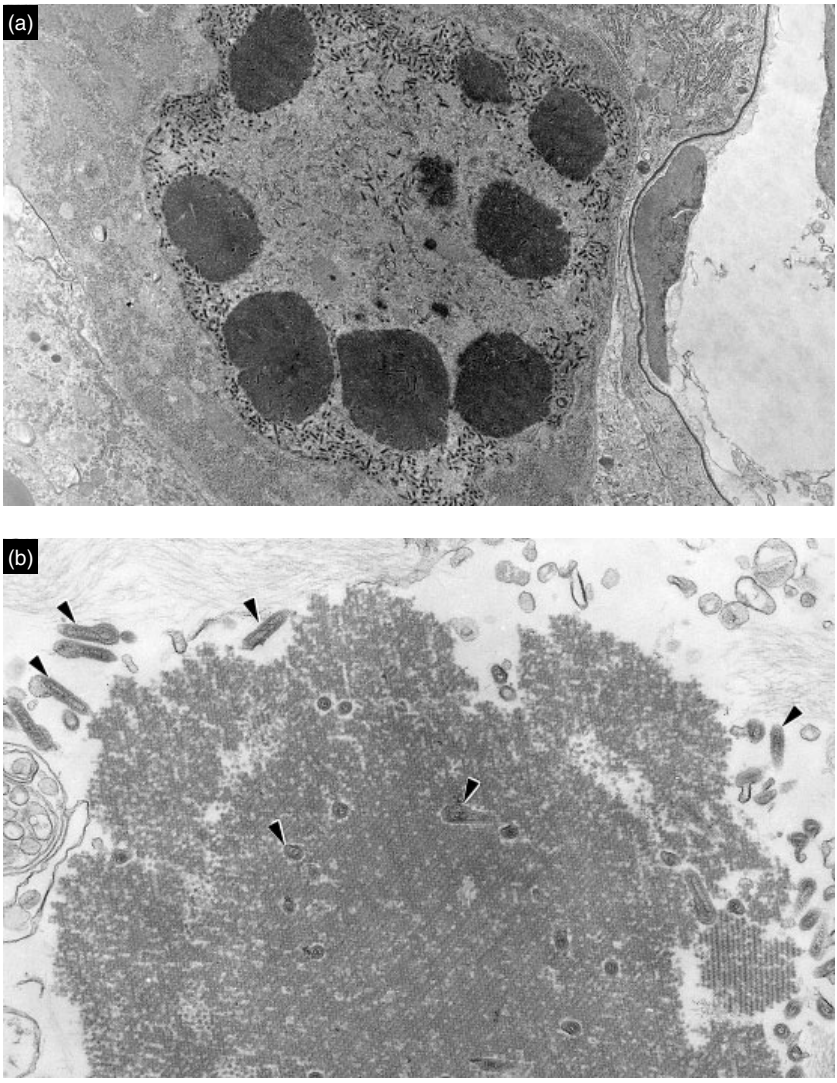


Fig. 20.6. (a) Monodon baculovirus (MBV) occlusion bodies in the nucleus of a hypertrophied hepatopancreas cell in *Penaeus monodon*. Photograph courtesy of D.V. Lightner (TEM, ×5940). (b) MBV virions (arrows) lying within and around the occlusion body of an infected hepatopancreas nucleus. Photograph courtesy of D.V. Lightner (TEM, ×33,333).

(overcrowding, nutrient build-up, transportation and handling) is believed to be a significant exacerbating factor (Brock and Lightner, 1990).

MBV is distinguished from BP by size and shape of the occlusion body. MBV occlusion bodies are <0.1 µm to nearly 20 µm in diameter and are roughly spherical (Lightner *et al.*, 1992b) (Fig. 20.6a,b). Virion and nucleocapsid sizes of BP and MBV overlap (~ 75 nm in diameter and 300 nm in length). Johnson and Lightner (1988) provide detailed ultrastructural descriptions of both types of baculovirus.

A third nuclear polyhedrosis baculovirus was isolated from fleshy prawn

(*P. chinensis* [*orientalis*]) suffering mass mortalities in Korea (Lee and Kim, 1994). Virions replicated in a *Spodoftera frugiperda* (Lepidoptera: Noctuidae) cell line and this was the first case of successful reproduction of an aquatic virion on an invertebrate cell line. The virus produced tetragonal or hexagonal polyhedra, which differed in shape from BP and MBV, but overlapped in size (3 μm in width). Johnson and Lightner (1988) cite, 'Gouranton (1972) pointed out, the ability of granulosis viruses ... to form occlusion bodies differs according to the cell-type infected'.

Thus, the difference between the tetragonal–hexagonal polyhedra of fleshy prawn and the subspherical polyhedra of MBV may not be distinct. Fleshy prawn virions (30–40 nm in diameter and 200–400 nm in length) overlap BP and MBV in size.

A fourth baculovirus, known as baculoviral midgut-gland necrosis virus (BMNV), was reported originally as a non-occluded (Type C) baculovirus (Sano *et al.*, 1981). Subsequent molecular analyses have redescribed the virus as PjNOB I (*P. japonicus* non-occluded baculovirus; first reported type) (Arimoto *et al.*, 1995). It affects hatchery-cultured kuruma shrimps (*P. japonicus*) from southern Japan and Korea, where it has been a chronic problem since 1971 (Sano *et al.*, 1984). Giant tiger prawn, fleshy prawn and green tiger prawn have also been infected experimentally. The same virus has been reported from giant tiger prawn and eastern king prawn in East and South-east Asia (Natividad and Lightner, 1992).

BMN virus infects the hepatopancreas of larval and postlarval shrimp, causing up to 98% mortality in PL-10 (10-day-old postlarvae) (Sano *et al.*, 1981). Early clinical signs are a 'white-turbid' or 'cloudy' hepatopancreas. Severely infected shrimp are inactive, float to the surface and are distinguished by a white midgut line (Sano *et al.*, 1984). Early names for the disease reflect these clinical signs: 'midgut gland cloudy disease', 'white turbid liver disease' and 'white turbidity disease'.

Infected hepatopancreas tissues may contain Gram-negative bacteria, but no occlusion bodies. As with BP and MBV, BMN causes marginated, reduced nuclear chromatin and nucleolar dissociation. Normal nuclei (approximately 10 μm diameter) are distinguished easily from infected nuclei (20–30 μm diameter) using routine histology. Host-cell hypertrophy, marginated chromatin and refractive chromatin are relatively accurate for presumptive diagnosis of BMN at patent, moderate or late stages of development (in conjunction with clinical signs). Diagnosis can be confirmed ultrastructurally or by indirect fluorescent antibody, which can detect infections as early as 18–24 h post-exposure (prepatent period) (Sano *et al.*, 1984). BMN virions are rod-shaped, 72×310 nm and closely resemble non-occluded MBV. No clinical infections have been detected in wild shrimp (Brock and Lightner, 1990).

Parvoviridae

Several parvovirus-like infections of hepatopancreas epithelia (hepatopancreatic parvo-like virus [HPV]; Lightner and Redman, 1985) of penaeids from Asia and the Americas are now recognized under one common name, infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Bell and Lightner, 1984; Lightner *et al.*, 1992b,c) and are grouped into a single genus, *Brevidensovirus*, in the family Parvoviridae (Fauquet *et al.*, 2005a). A single species designation, PstDNV (*P. stylirostris* densovirus), contains three IHHNV genotypes (Fig. 20.7a–c):

- Type 1 – from the Americas and East Asia (primarily the Philippines)
- Type 2 – from South-east Asia
- Type 3a – from East Africa, India and Australia
- Type 3b – from the Western Indo-Pacific (Madagascar, Mauritius and Tanzania).

Types 1 and 2 cause disease in Pacific blue, giant tiger and white-leg shrimp, known as 'runt deformity syndrome' (RDS). Types 3a and 3b are found inserted into the genome of giant tiger shrimp and do not appear to be infectious (Tang and Lightner, 2006; Tang *et al.*, 2007).

Clinical signs of infection are poor growth, surface fouling, atrophy of the

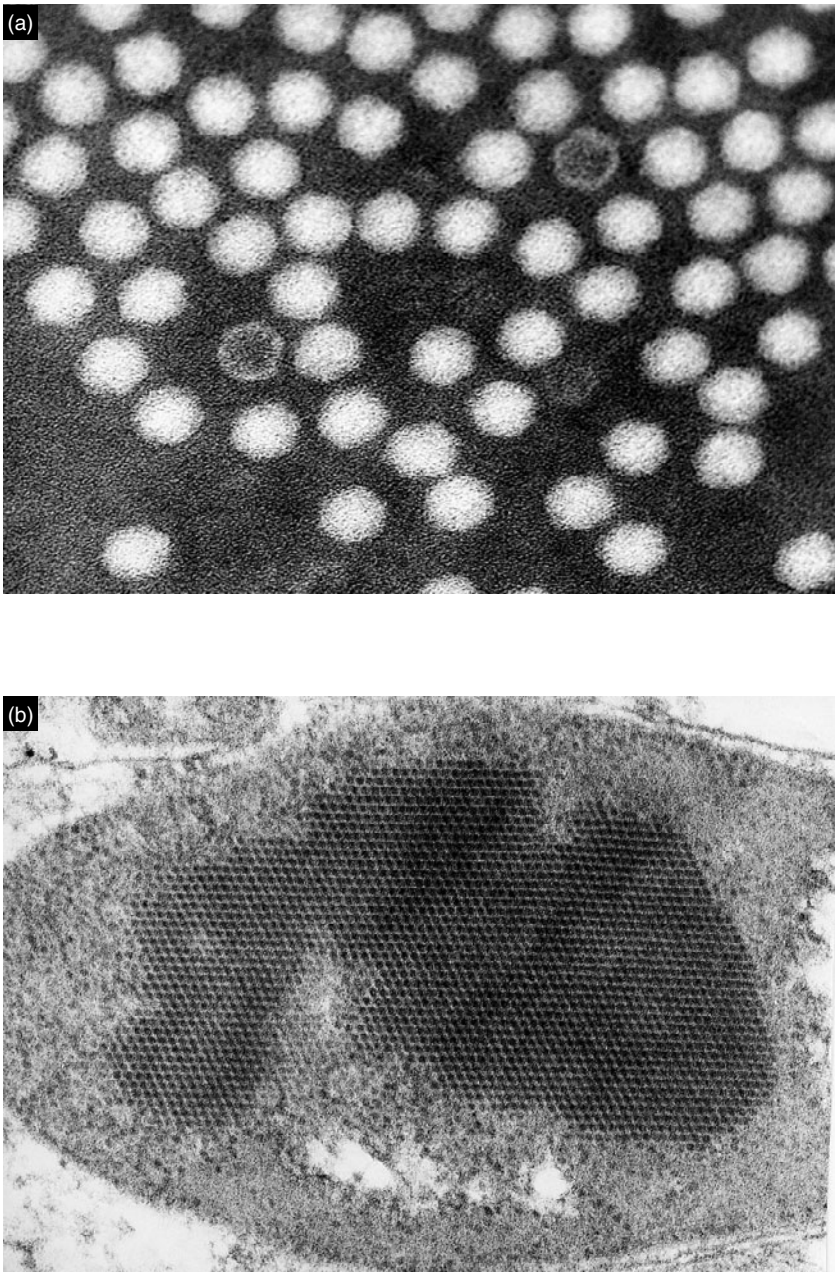


Fig. 20.7. (a) Infectious hypodermal and haematopoietic necrosis (IHHN) virions purified from *Penaeus stylirostris*. Photograph courtesy of D.V. Lightner (negative stain, ×462,000). (b) Paracrystalline array of IHHN virions in an infected epithelial cell of *Penaeus stylirostris*. Photograph courtesy of D.V. Lightner (TEM, ×100,000).

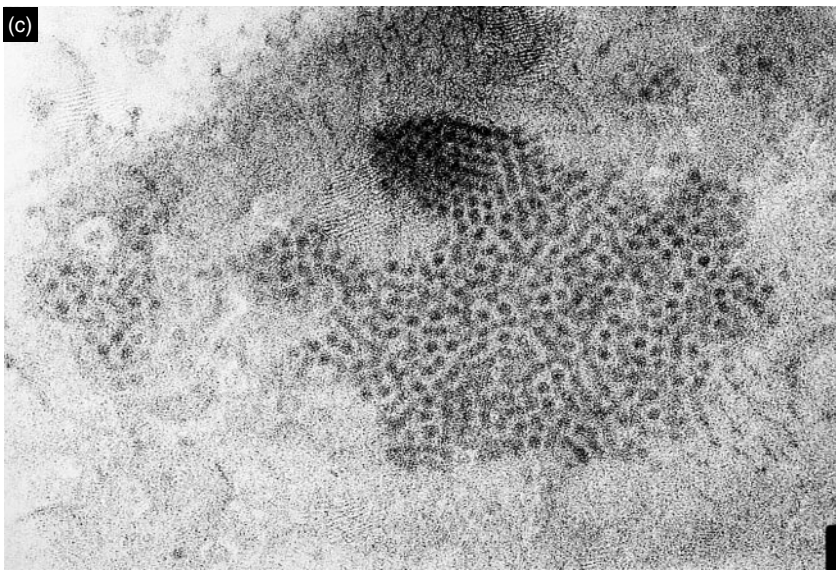


Fig. 20.7. (c) IHHN virions in a partial paracrystalline array in an antennal gland cell of *Penaeus stylirostris*. Photograph courtesy of D.V. Lightner (TEM, ×100,000).

hepatopancreas and opacity of the abdominal musculature (Lightner and Redman, 1985). As with *Reovirus*-like infections, these signs are non-specific and frequently complicated by multiple infections (Lightner *et al.*, 1992b). Mortalities are higher in juveniles than younger stages, with 100% cumulative mortality in white shrimp (*P. merguiensis*) within 4–6 weeks of the appearance of clinical signs (Lightner and Redman, 1985). In contrast, a heavy infection was detected in a single, apparently healthy brown tiger shrimp (Paynter *et al.*, 1985, cited in Lightner *et al.*, 1992b).

Infected hepatopancreatic tubule epithelia show displaced chromatin and hypertrophy due to Feulgen-positive intranuclear inclusions (Fig. 20.8a). In larval fleshy prawn, these inclusions occur predominantly in the distal tubules of the hepatopancreas, as well as the anterior midgut. Infected tissues are atrophied but show minimal necrosis or haemocyte infiltration. The intranuclear inclusions consist of granular virogenic stromae and isometric HPV virions, 22–24 nm in diameter (Lightner and Redman, 1985) (Fig. 20.8b). Particle size, Feulgen-positive histochemistry and intranuclear replication from virogenic stromae containing microfibrils are all features of the Parvoviridae (Francki *et al.*, 1991).

Totiviridae

A relatively recently recognized disease, known as 'infectious myonecrosis' (IMN), has emerged in farmed populations of white-leg shrimp in north-eastern Brazil (Lightner *et al.*, 2004; Nunes *et al.*, 2004; Poulos *et al.*, 2006; Andrade *et al.*, 2007) and South-east Asia (Saengchan *et al.*, 2007). The disease appears to be related to stressful growing conditions and is attributed to a dsRNA non-enveloped 40 nm icosahedral virus assigned to the Totiviridae (Fauquet *et al.*, 2005c; Poulos *et al.*, 2006; Nilbert, 2007). Experimental infections to determine host specificity have been successful in Pacific blue shrimp and giant tiger shrimp, and an assessment of transmission risk via trade has resulted in the listing of IMN as a reportable disease by the OIE in

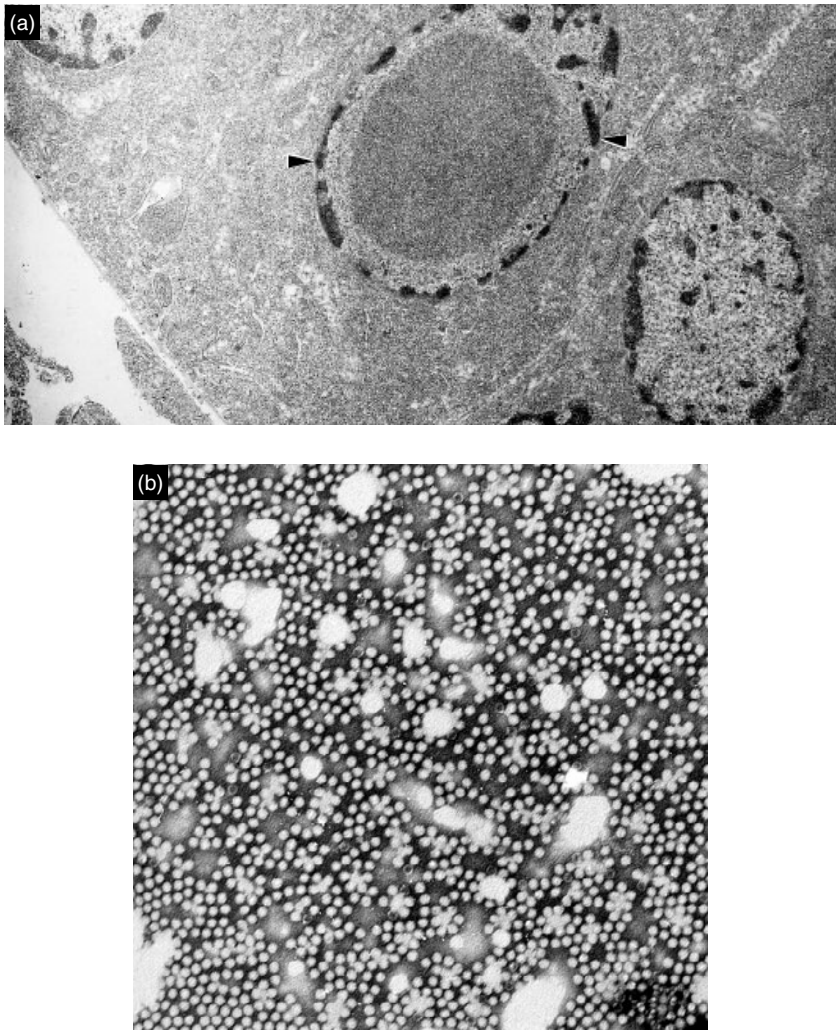


Fig. 20.8. (a) Hepatopancreatic parvo-like virus (HPV) in the nucleus of a hepatopancreatic epithelial cell of *Penaeus chinensis*. Note marginated chromatin of infected nucleus (arrow). Photograph courtesy of D.V. Lightner (TEM, ×9600). (b) Purified HPV virions from *Penaeus chinensis*. Photograph courtesy of D.V. Lightner (negative stain, ×105,000).

2007. Clinical signs of whitening of the tail musculature are similar to those of 'white tail disease' found in giant river prawn (*Macrobrachium rosenbergii*); however, this disease is caused by a nodavirus (MrNV) and is described below.

White-leg shrimp are the species most susceptible to infection, which is characterized by sudden high mortalities following a significant change in temperature or salinity, or handling, especially in early juvenile, juvenile or subadult feeding stages in marine or low brackish production environments. In acute infections, shrimp show focal to extensive white discoloration of the abdominal skeletal muscle, especially in the distal abdominal segments and tail fan, which may become reddened or necrotic. In addition to

the striated musculature, other tissues that may be infected are the connective tissues, haemocytes and paraenchymal cells of the lymphoid organ (Lightner *et al.*, 2004). Exposure of the paired lymphoidal organs shows a three to four times greater than normal hypertrophy, characteristic of IMN versus other viral infections of penaeid shrimp.

Transmission is horizontal via cannibalism (Poulos *et al.*, 2006), although it is also suspected that disease spread within and between farms may be facilitated by seabird faeces or regurgitated shrimp containing viable virions (Vanpatten *et al.*, 2004). Survivors of infections that are used as broodstock are also suspected of being potential sources of 'vertical' (surface contamination) transmission to their progeny. Although this transmission route has yet to be demonstrated clearly, surface disinfection of eggs and larvae is recommended as a good husbandry practice in areas endemic for IMN (Chen *et al.*, 1992).

Dicistroviridae

Taura syndrome virus (TSV) causes disease in a wide range of penaeids, but the most susceptible species are white-leg and Pacific blue shrimp (Lightner, 1996a,b; Bonami *et al.*, 1997; Brock, 1997; Mari *et al.*, 1998). Although associated originally with aerial spraying of agricultural fungicides (with putative moult-inhibiting sterols) in the Taura region of Ecuador in 1992 (Intriago *et al.*, 1997), the syndrome quickly was related to infection by a non-enveloped single-strand RNA virus (30–32 nm in diameter) which spread throughout most shrimp farming areas of the Americas and Hawaii (Hasson *et al.*, 1995; Bonami *et al.*, 1997; Brock, 1997; Aguirre-Guzman and Ascencio-Valle, 2000; Lightner, 2005). It has been documented from wild stocks of white-leg shrimp on the Pacific coasts from Mexico to Peru (Lightner *et al.*, 1995; Lightner and Redman, 1998); however, no wild reservoirs have been found, to date, along the Atlantic coasts of shrimp producing countries in the Americas (Hasson *et al.*, 1999a, Lightner, 1996a,b, 2005) where the disease is reported in cultured stocks. In 1999, infected shrimp were imported into Chinese Taipei and TSV has now spread to China, Thailand, Malaysia and Indonesia, resulting in several major epizootics with some losses of over 90% (Chang *et al.*, 2004; Neilsen *et al.*, 2005; Tang and Lightner, 2005).

At least four genotypes of TSV particles have been described based on structural capsid protein VP1 (Bonami *et al.*, 1997; Mari *et al.*, 1998, 2002):

1. The Americas group;
2. The South-east Asian group;
3. The Belize group (Erickson *et al.*, 2002, 2005); and
4. The Venezuelan group.

TSV was listed recently as an unassigned species in the Family Dicistroviridae by the ICTV (Fauquet *et al.*, 2005b).

There are three distinct phases of TSV infection: acute, transition (recovery) and chronic. Acute phase infections usually hit small juveniles 14–40 days post-stocking in nursery or grow-out stages of production; however, larger juveniles and adults can become infected if not previously exposed to the virus. Infected shrimp show overall reddish discoloration, empty guts, softened cuticles and distinct reddening of the tail fan and pleopods. They congregate at pond edges or surfaces due to hypoxia, which can attract seabirds as the first alert to the production manager of a disease problem. Most shrimp in this phase of TSV infection do not survive moulting. Histologically, necrotic lesions are present in the cuticular epithelium, appendages, gills, gut, subcuticular connective tissues and adjacent striated muscle fibres. There is no significant inflammatory response to these lesions in the acute stage of infection (Brock *et al.*, 1995, 1997; Lightner *et al.*, 1995; Brock, 1997; Hasson *et al.*, 1999b).

In contrast, the transition/recovery phase of TSV infection demonstrates fewer cuticular lesions and marked haemocyte infiltration of areas of tissue necrosis. Melanization of the haemocyte response site gives rise to black tissue spots that characterize this stage of the disease. Damaged cuticle and subcuticular tissues may show signs of secondary infection (Hasson *et al.*,

1999b). Molecular examination (*in situ* hybridization) of the lymphoidal organs of shrimp at this stage reveals large concentrations of TSV.

The chronic stage of infection is characterized by no gross signs of infection. Histologically, TSV infection can be detected only by the presence of large numbers of prominent lymphoidal organ 'spheres', which may remain associated with the lymphoid organs or detach and get lodged elsewhere in the haemocoel (Hasson *et al.*, 1999b).

Simple bioassays are used with naive (specific pathogen free [SPF]) white-leg shrimp to confirm whether or not shrimp showing no clear evidence of infection are carriers of TSV. *Per os* or inoculation of naive shrimp produces clinical signs and mortality within 3–8 days (Srisuvan *et al.*, 2006).

Reo-like viruses

Reovirus-like particles were found in cytoplasmic inclusions in the hepatopancreas of juvenile kuruma shrimp imported into France from Japan (Tsing and Bonami, 1987) and in giant tiger shrimp from Hawaii (Lightner *et al.*, 1985) and Malaysia (Nash *et al.*, 1988). 'Gut and nerve syndrome' (Lightner *et al.*, 1984; Lightner, 1988a), which causes mortalities in kuruma shrimp in Hawaii, has been linked putatively to this virus, but this has not been confirmed (Brock and Lightner, 1990).

Krol *et al.* (1990) reported *Reo*-like viruses in captive white-leg shrimp from the southern USA. The shrimp were infected experimentally with BP, but subsequent examination revealed *Reovirus*-like particles in the same host, and even the same cell, as BP. The *Reovirus*-like particles resemble those described from kuruma and giant tiger shrimp.

Since all the *Reovirus*-like infections were associated with BP, Krol *et al.* (1990) speculated introduction with the BP experimental infection. Alternatively, the former virus may only manifest itself during the stress of BP infection. Determination of the exact pathogenic effect of the reovirus-like virus is further complicated by other infections (MBV, rickettsial and Gram-negative bacteria) of the anterior R- (Restzellen) and F- (Fibrillenzellen) midgut cells (Nash *et al.*, 1988; Brock and Lightner, 1990; Lightner and Redman, 1992). Clinical signs are poor feeding, surface fouling, cessation of burying in the sand and reddish discoloration of the telson and uropods (Tsing and Bonami, 1986, 1987). Some shrimps show no clinical signs, despite being infected (Tsing and Bonami, 1987).

Roniviridae

Yellow-head disease (YHD) in giant tiger shrimp from Thailand, originally described as a Type B baculovirus (Chantanachookin *et al.*, 1993), has caused acute epizootics in PL-20 and subadult shrimps in Thailand since 1990 (Boonyaratpalin *et al.*, 1993). Early clinical signs include erratic swimming, floating near the surface, yellowing of the cephalothorax and gills and general bleaching of body colour. The distinctive yellow head (carapace) discoloration is due to lightening of the hepatopancreas from its healthy olive-brown colour. Cumulative mortalities can reach 100% within 3–5 days following the appearance of clinical signs.

Spherical, or irregular-shaped, Feulgen-positive inclusion bodies occur in the haemocytes, haematopoietic tissues, lymphoid organ, gills, cuticle and fixed phagocytes of the heart (Fig. 20.9a). Epidermal necrosis may be evident in the gills and cuticle of the body. Virogenesis occurs in the cytoplasm, and the rod-shaped virions (40–50×150–200 nm) have a single envelope (Fig. 20.9b–d). Occasional intranuclear replication has also been noted, with virions budding through the nuclear membrane (Boonyaratpalin *et al.*, 1993). Although described originally as a non-occluded baculovirus (Boonyaratpalin *et al.*, 1993), granulosis-type morphology was also observed (Chantanachookin *et al.*, 1993) and attributed to a putative Rhabdovirus, Picornavirus or Coronavirus. It also demonstrated ultrastructure, biochemical and histopathology attributes similar to those described for gill-associated virus (GAV) infections in healthy giant tiger shrimp in

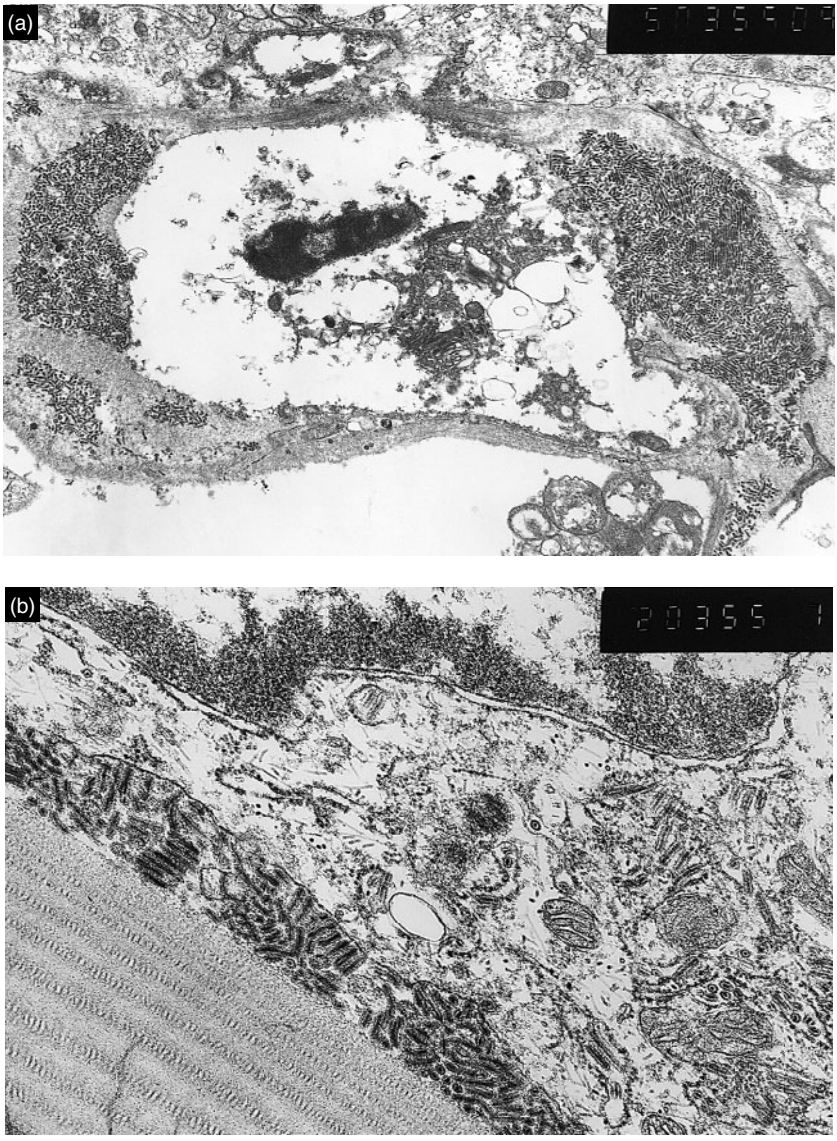


Fig. 20.9. (a) Yellow-head baculovirus (YHB) in the cytoplasm of an infected lymphoid organ in *Penaeus monodon*. Photograph courtesy of S. Boonyaratpalin (TEM, ×7700). (b) Gill lamellae of *Penaeus monodon* infected by YHB. Note aggregations of viral particles under the cuticle of the gill lamella. Photograph courtesy of S. Boonyaratpalin (TEM, ×30,800).

Australia (Spann *et al.*, 1998, 2000; Walker *et al.*, 2001; Tang *et al.*, 2002; Wijegoonawardane *et al.*, 2008) and lymphoid organ virus (Spann *et al.*, 1995; Lightner, 1996a). The viruses involved in these detections were enveloped, single-stranded RNA (Wongteerasupaya *et al.*, 1995), rod-shaped with distinctive envelope ornamentation by peplomer projections 11 nm from the envelope surface. Analysis of structure,

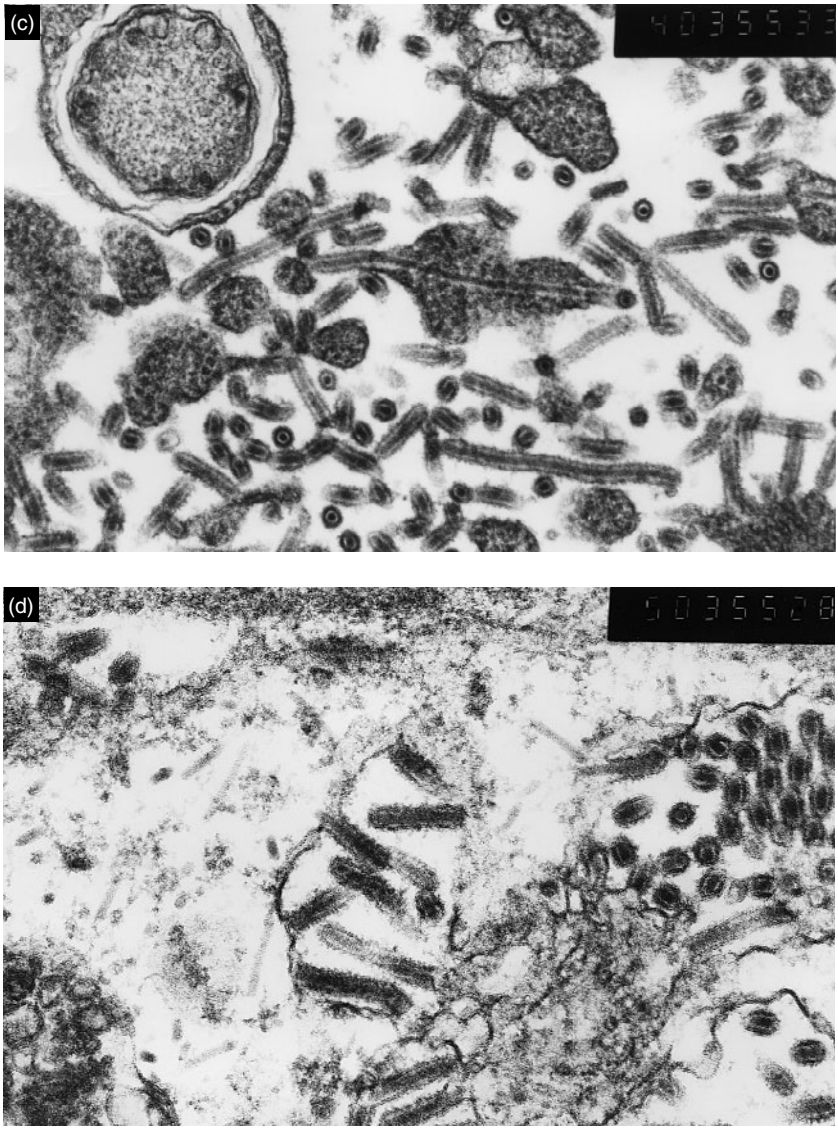


Fig. 20.9. (c) Non-occluded YHB virions in the lymphoid organ of *Penaeus monodon*. Photograph courtesy of S. Boonyaratpalin (TEM, ×61,600). (d) Non-occluded YHB virions in the lymphoid organ of *Penaeus monodon*. Photograph courtesy of S. Boonyaratpalin (TEM, ×77,000).

nucleoprotein and glycoprotein components led to the taxonomic determination that these viruses were related to the Order Niridovirales. The eighth report of the ICTV places both GAV and YHD in the same species (GAV) within a new genus *Okavirus* and new family Roniviridae in the order Niridovirales (Walker *et al.*, 2004).

The yellow-head complex comprises six genotypes, of which YHD (genotype 1) is

the only one responsible for yellow-head disease. GAV (genotype 2) and four other related genotypes (3–6) are found in giant tiger shrimp in East Africa, Asia and Australia, but rarely are associated with disease (Walker *et al.*, 2001; Wijegoonawardane *et al.*, 2008).

YHD outbreaks have been reported solely from cultured populations of giant tiger shrimp in Thailand, the People's Republic of China, Chinese Taipei, India, Indonesia, Malaysia, the Philippines, Sri Lanka and Vietnam (Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993; Lightner, 1996a; Wang *et al.*, 1996; Albaladejo *et al.*, 1998; Mohan *et al.*, 1998; Wang and Chang, 2000; Wijegoonawardane *et al.*, 2008; Sanchez-Barajas *et al.*, 2009); however, natural infections have also been detected in kuruma, banana, Pacific blue and white (*P. setiferus*) shrimp and Antarctic krill (*Euphausia superba*), which are assumed to be natural reservoirs of infection. Preparations from paste shrimp (*Acetes* sp.) collected from infected ponds also appear capable of transmitting the disease to naive giant tiger shrimp (Flegal *et al.*, 1995). Infections have also been detected in cultured white-leg shrimp from Mexico (Sanchez-Barajas *et al.*, 2009).

Horizontal transmission has been well demonstrated from field observations as well as experimental infections that have been induced in a wide range of other penaeid and palaemonid shrimp. Laboratory analyses of susceptibility have shown high mortality in giant tiger shrimp, white-leg shrimp, Pacific blue shrimp, brown shrimp (*P. aztecus*), pink shrimp (*P. duorarum*) and *P. styliferus*; Sunda river prawn (*M. sintangense*) and barred estuarine prawn (*Palaemon serrifer*) (Lu *et al.*, 1994, 1997; Lightner *et al.*, 1998; Longyant *et al.*, 2005, 2006). Sixteen crab (Brachyuran) species collected from around shrimp farms in Thailand showed no evidence of YHD (Longyant *et al.*, 2006), so do not appear to be reservoirs of infection. Vertical transmission via surface-contaminated gametes or infected gametogenic tissues is also suspected (Cowley *et al.*, 2002).

Nodaviridae

A new disease has emerged recently in hatchery-reared giant river prawn that has been related to an unusual association between a nodavirus (*Mr*NV – *M. rosenbergii* nodavirus) and a 'satellite' virus designated, somewhat unscientifically, as XSV (extra small virus) (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a). Infection with this pair of viruses causes a condition known as white tail disease (WTD) or white muscle disease (WMD) in larval/postlarval and early juvenile stages of the freshwater prawn. WTD has been responsible for mass mortalities in culture systems in the French West Indies (Arcier *et al.*, 1999), the People's Republic of China (Qian *et al.*, 2003), Chinese Taipei (Wang *et al.*, 2007), India (Sahul Hameed *et al.*, 2004b) and Thailand (Yoganandhan *et al.*, 2006). The severity of the disease has led to its addition in 2007 to diseases listed by the OIE as being of trade and national disease control significance (OIE, 2009a,b).

*Mr*NV is a non-enveloped, five- to six-sided particle with a diameter of 26–27 nm and is recognized by the ICTV as a member of the Nodaviridae. Its role in WTD is well documented. The taxonomic relationship and pathogenic role of the XSV is less well understood. XSV is also non-enveloped and five- to six-sided, but measures 14–16 nm. This is the first documented case of a satellite–nodavirus association (Bonami *et al.*, 2005). Satellite viruses depend on co-infection of a host cell for productive multiplication. Their nucleic acids have nucleotide sequences that are distinct from the helper virus or host. Fauquet *et al.* (2005d) cite bee paralysis virus and tobacco necrosis virus diseases as examples of animal and plant viral associations.

Freshwater prawn infections target gill tissues, head and abdominal musculature, heart, ovaries, pleopods and tail muscle (Sri Widada *et al.*, 2003; Sahul Hameed *et al.*, 2004a). Transmission is reported as vertical (trans-ovum) based on ovarian infections, as well as horizontal (Qian *et al.*, 2003; Sahul Hameed *et al.*, 2004a; Sudhakaran *et al.*, 2006). Mortality rates can be as high as 95%;

however, postlarvae that survive are believed to act as carriers for the rest of their lives (Qian *et al.*, 2003; Sahul Hameed, *et al.*, 2004a). The white discoloration associated with WSD is not disease specific and molecular tools or ultrastructural examination of affected tissue is required for definitive diagnosis. Interestingly, the viruses are propagated easily in the C6/36 mosquito, *Aedes albopictus*, cell line, as well as the striped snakehead (*Ophicephalus striatus*) cell line, SSN-1 (Sudhakaran *et al.*, 2007). In addition, several diverse species have been identified as vectors of the disease: shrimps – Indian white shrimp (*P. indicus*), giant tiger shrimp and kuruma shrimp (Sudhakaran *et al.*, 2006); brine shrimp (*Artemia* spp.) and aquatic insects – giant water bugs (*Belostoma* sp.), dragonfly nymphs (*Aesohna* sp.), diving beetles (*Cybister* sp.) and back-swimmers (*Notonecta* sp.) – based on positive RT-PCR results (Sudhakaran *et al.*, 2008).

The unsual viral association, pathology, cell-line isolation and propagation, and transmission of WTD in cultured giant river prawn suggest a possible transfer of a relatively benign wild infection into a less benign infection under intense culture conditions.

Viruses of Brachyura Latreille, 1803

At least 26 types of virus have been reported from crabs, principally from wild populations where infections are sporadic or subclinical (see reviews by Johnson, 1983, 1984; Brock and Lightner, 1990; Messick and Sindermann, 1992; Stewart, 1993). Since the previous review of microbial diseases, little additional work has been done on viral diseases of crabs. This may reflect the burgeoning need for penaeid shrimp virology research due to the emergence of several serious diseases over the past 10 years (described above) which have had a significant socio-economic impact on shrimp aquaculture development. It should also be noted that the diseases described below do not include infections described from shrimp, which have also been found in crab species.

Baculoviruses and baculo-like viruses

A non-occluded baculovirus named Tau, after the lagoon in southern France from which infected European portunid crabs (*Carcinus mediterraneus*) were collected (Pappalardo and Bonami, 1979), induced 100% mortality in exerimentally inoculated crabs (Pappalardo *et al.*, 1986). Infections developed faster at 23°C than at 18°C and crabs fed infected tissues developed heavier infections than those which were inoculated. Clinical signs of Tau infections were non-specific: increased lethargy, decreased feeding and death within a few days of experimental infection.

The Tau virus infects the hepatopancreas epithelia, causing nucleus hypertrophy and marginated chromatin. Negative stain preparations show rod- or bow-shaped virions 80–90×340–380 nm with nucleocapsids measuring 60–70×300–320 nm. Tail-like extensions were demonstrated with negative staining (Pappalardo and Bonami, 1979). A second non-occluded bacuolvirus, Tau 2, was reported by Mari and Bonami (1986) from the hepatopancreas of the European shore crab (*C. maenas*); however, no further information on this virus has been published (Brock and Lightner, 1990).

A third non-occluded baculo-like virus, found in the haemocytes and connective tissue cells of regenerating limb buds of European shore crab, was characterized by small aggregations of virions in hypertrophied nuclei with marginated chromatin (Bazin *et al.*, 1974). Mari and Bonami (1986) named the virus B1 and compared it with a similar virus, B2, from the haemocytes of Mediterranean shore crab. B1 particles are rod-shaped with nucleocapsids 75–80×230–280 nm. B2 particles are 70–80×280–320 nm, with enveloped stages 90–110×340–380 nm. B2 virions are dispersed through the nucleoplasm and, as with B1, no gross pathology was observed. Slightly larger baculo-like virions (90–100 nm wide and 300–320 nm long) have also been reported from the

European shore crab (Bazin *et al.*, 1974), but their relationship to B1 and B2 and their pathogenicity have not been described.

Johnson (1988a) found a similar non-occluded baculovirus in the haemocytes and haematopoietic tissue of a single (n=1/74) European shore crab collected from Woods Hole, Massachusetts, USA. The RV-CM (rod-shaped virus from *C. maenas*) infection was associated with milky haemolymph and abnormal clotting characteristics; however, the specific pathology was complicated by concurrent infections with a *Rhabdo*-like virus, similar to EHV (enveloped helical virus) of blue crab (*Callinectes sapidus*) (see *Rhabdo*-like viruses, below). Nuclei infected by RV-CM are Feulgen positive but less hypertrophied than with other baculovirus infections. The histopathology resembles that of baculo-B infections in blue crab (Johnson, 1988a). RV-CM has rod-shaped nucleocapsids, 95–110×190–540 nm, which are dispersed throughout the nucleoplasm. Mature virions assume a bent or V-shaped appearance (Johnson, 1988a), similar to that of B1, in European shore crab (Bazin *et al.*, 1974).

Herpes-like viruses

At least three hexagonal, intranuclear viruses from crabs fall within the size range of *Herpes*-like viruses. All were described as *Herpes*-like; however, the taxonomic affinities of two are under debate.

A *Herpes*-like virus (HLV) found in immature blue crab from Virginia, USA, (Johnson, 1976) was renamed bi-facies virus (BFV) due to subsequent detection of two developmental forms (Johnson, 1988a). Under laboratory conditions, infected crabs appear normal until shortly before death, when they become inactive and stop feeding. The haemolymph loses its clotting properties and becomes opaque. Crabs injected with haemolymph from moribund specimens died 30–40 days post-injection (Johnson, 1984).

BFV infects the haemocytes, but is found occasionally in haematopoietic and, more rarely, connective tissue as well as gill epithelia. Infected nuclei are hypertrophied, Feulgen positive and have marginated chromatin. Feulgen-negative inclusions may also be present in the nuclei and cytoplasm, but do not contain virions. Virogenesis is intranuclear (Johnson, 1988a).

BFV Type A particles are icosahedral, approximately 197–233 nm in diameter and contain a nucleoprotein core (75×138 nm) surrounded by an electron dense toroid and two membranes (Johnson, 1988a). Type B particles are approximately 174–191 nm in diameter and have a slightly longer nucleoprotein core (70×156 nm) than Type A, with a single, irregular, circular or elliptical membrane (Johnson, 1988a). BFV Type A is similar in appearance to the *Herpes*-like virus in American oysters (*C. virginica*), described by Farley (1978), and in other bivalve hosts, as described by Hine *et al.* (1992). Both BFA types occur together, lying loose in the cytoplasm.

Another *Herpes*-like virus was detected during an investigation of dwindling blue and red king crab (*Paralithodes platypus* and *P. camtaschatica*, respectively) stocks off Alaska (Sparks and Morado, 1986). Hypertrophied nuclei, marginated chromatin and eosinophilic intranuclear inclusions were found in the bladder epithelium, antennal gland or, less frequently, the hindgut epithelium. The inclusion bodies were pleomorphic and embedded in a fine granular, Feulgen-negative (or weakly Feulgen-positive) substance. When few nucleocapsids were present, the inclusion bodies had well-defined edges; however, as numbers increased, the edges became 'frayed' in appearance (Sparks and Morado, 1986). The nucleocapsids were non-enveloped, hexagonal, with an electron dense core (55–60×90–105 nm) surrounded by a less electron dense substance 90–195 nm in diameter, plus two membrane-like layers (tegument). The capsids, approximately 140–165 nm in diameter, resembled BFV Type A, although slightly smaller. A similar virus was also reported by the same authors in golden king crab (*Lithodes aequispina*). No correlation between the viral infections and the declines in crab populations could be determined conclusively (Sparks, 1985).

Parvo-like viruses

A parvo-like virus, PC84, infects the connective tissue of Mediterranean shore crabs and is linked to experimentally induced mortalities (Mari and Bonami, 1986). The chromatin of infected nuclei is replaced by an amorphous, Feulgen-positive material containing numerous PC84 virions. These are also found in the cytoplasm and in paracrystalline arrays in intercellular spaces. PC84 is non-enveloped, icosahedral, 23–27 nm in diameter (TEM) or 29–31 nm (negative stain preparations) and appears to have double-strand DNA virus characteristics, with a molecular weight of 1.4×10^6 daltons (Mari and Bonami, 1988). The significance of PC84 to wild populations of the Mediterranean shore crab remains unknown.

Reo-like viruses

P-virus (paralysis virus) was the first viral infection to be detected in crustaceans (Vago, 1966). It was found in portunid crabs (*Macropipus depurator*) from southern France. Experimental infections with haemolymph and purified virus resulted in leg trembling in over 60% of crabs within 6 days post-inoculation. Paralysis progressed up to day 9 and there was slight darkening of the exoskeleton in infected crabs (Vago, 1966; Bonami, 1973). Experimental infections have resulted in 70–85% mortality, but the significance of P-virus to wild populations is unknown.

P-virus infects non-epithelial cells of the gills, hepatopancreas and midgut connective tissue. Although the paralytic signs implicate nervous tissue involvement, investigation of these tissues has not been reported (Johnson, 1983). Paracrystalline inclusions, 5–10 μm in diameter, occur in the cytoplasm and contain icosahedral, non-enveloped virions, 58–65 nm (negative staining) in diameter, which are believed to be ribonucleic (Bonami *et al.*, 1976). P-virus was found in conjunction with S-virus (see Bunya-like viruses below), sometimes in the same tissues but not the same cell. Combined and separate experimental infections resulted in similar clinical disease and high mortality (Bonami, 1973).

Another reo-like virus (RLV) was reported in laboratory-reared blue crab from the eastern USA; however, the effect on wild populations is unknown (Johnson and Bodammer, 1975; Johnson, 1988b; Messick and Kennedy, 1990). Concurrent infections by other viruses, as well as an unidentified microsporidian (Johnson, 1977; Johnson and Farley, 1980), complicated the determination of specific RLV pathogenicity in natural infections. Experimental infections indicated a synergistic effect between RLV and a rhabdo-like virus (RhVA – see below) in the development of pathology (Johnson, 1983). Gross clinical signs were trembling, disorientation, paralysis, cessation of feeding, gill fouling and disruption of the normal moult cycle. Reduced clotting of the haemolymph was also noted (Johnson, 1984).

Infected haemocytes and haematopoietic tissues show signs of hypertrophy, necrosis, abnormal mitosis or multinucleosis. The cytoplasm is opaque and may contain densely basophilic, Feulgen-negative inclusion bodies. Tissue damage is concentrated in the gill epithelium, nerve and brain tissue; however, the interstitial tissue of the hepatopancreas may also show vacuolization and necrosis (Johnson, 1984). Non-enveloped, icosahedral RLV virions, 55–60 nm in diameter, may also form cytoplasmic paracrystalline arrays. Morphology, cytoplasmic virogenesis and Feulgen-negative (RNA) histochemistry led to the *Reovirus*-like designation by Johnson and Bodammer (1975).

An unnamed *Reovirus*-like infection was also reported in the Mediterranean shore crab from Sète, France (Bonami, 1976). Gill epithelia were destroyed in crabs inoculated with purified virus, or exposed to infected tissues. Death occurred within 8 days of experimental exposure; however, the effect on wild populations is not known (Mari and Bonami, 1986; Brock and Lightner, 1990). The virions are non-enveloped, icosahedral, 55 nm in diameter and form distinctive six-particle rosettes (Mari and Bonami, 1986).

A similar virus reported from the same host by Mari and Bonami (1986) was named W2. Infections occurred in the haemocytes and interstitial cells of the hepatopancreas, midgut and gills, where six-particle rosettes were formed by virions 55–60 nm (ultrathin section) or 65–70 nm (negative stain) in diameter. The pathogenicity of W2 and features distiguishing it from the unnamed *Reovirus* were not reported.

Picorna-like viruses

Chesapeake Bay virus (CBV), or blue crab picorna-like virus, was detected in juvenile blue crab being used for nutritional studies (Johnson, 1978). Most of the crabs died over a 2 month period, but the specific pathogenic effect of the picorna-like virus could not be determined due to the presence of other viruses (RhVA, EHV and RLV). The diseased crabs were often blind, exhibited erratic swimming behaviour and moulting patterns and rested head-down (Johnson, 1978). The significance of CBV (and the other viruses) to wild populations of blue crabs has yet to be determined.

Infections occur in the retina, gill, stomach, hindgut and general epidermis, antennal gland and bladder epithelia. Entire gill epithelia or retinal tissues can be destroyed. Haemocytes and haematopoietic tissues may also be infected and are characterized by hypertrophy and Feulgen-negative cytoplasm. Non-enveloped, icosahedral, 30 nm diameter virions undergo replication in the cytoplasm, where paracrystalline arrays may be formed. Interestingly, spikes were observed on the surfaces of the mature viral particles (Johnson, 1983), a feature not present in picornaviruses of vertebrates and insects (Francki *et al.*, 1991).

An association between two, apparently distinct, picorna-like viruses was reported from moribund portunis crabs (Bonami, 1976). The viruses, named V31–24 complex (or F-N virus), were non-enveloped, paraspherical and differentiated by size (24 and 31 nm diameter). Cytoplasmic infections occurred in the haemocytes and connective tissue cells. Samples of each virus were seperated by gel electrophoresis and inoculated into healthy crabs. Each caused morbidity (Bonami, 1976). The effect on wild populations is unknown.

Rhabdo-like viruses

A rhabdo-like virus of blue crab, originally reported by Jahromi (1977) and redescribed by Yudin and Clark (1978, 1979) as ecdysal gland virus-2 (EGV-2), was further redescribed by Johnson (1983) as rhabdovirus-A (RhVA), since the particles occurred primarily in the mandibular organ, rather than in the ecdysal gland. RhVA infects crabs from Maryland, Virginia and Texas (Yudin and Clark, 1978; Johnson, 1988b; Messick and Kennedy, 1990) and may have a broad distribution over the geographic range of the blue crab. Infected crabs do not generally show signs of disease until stressed by transportation, artificial holding conditions, eye-stalk ablation or concurrent infection by another virus, e.g. RLV, EHV, CBV, BFV and baculo-B (Johnson, 1983, 1984). Experimental infections with various combinations of these viruses demonstrated a synergistic pathogenic effect with RhVA and RLV infections (Johnson, 1983).

In addition to the mandibular organ, RhVA infects a wide range of tissues: Schwann cells, endothelial cells, haemocytes in the gastric mill muscles, connective and reserve tissues, nerve tissue, bladder epithelium, haematopoietic and epicardial tissues. Glial cells appear to be more heavily infected than other cells. The virions are predominantly bacilliform, 20–30×110–170 nm, although longer, filamentous forms (<600 nm in length) also occur (Johnson, 1983). These aggregate in nuclear membrane cisternae, the endoplasmic reticulum and may also form extracellular parallel arrays perpendicular to the plasmalemma.

Blue crab from the Gulf of Mexico have a second rhabdo-like virus which was described originally as ecdysal gland virus 1 (EGV-1) by Yudin and Clark (1978, 1979) but renamed RhVB (rhabdo-like virus B) by Johnson (1983) for the same reason as RhVA. No clinical signs have been attributed to RhVB infections, which occur primarily

within the mandibular gland. RhVB is differentiated from RhVA by morphology: RhVB measures 50–70×100–170 nm and is more ovoid than the bacilliform/filamentous forms of RhVA. Mature RhVB virions aggregate beneath the basal lamina of the mandibular organ and have surface spikes (Yudin and Clark, 1978). There have been no further reports of RhVB since the original description.

Bunya-like viruses

A bunya-like virus, described as crab haemocytopenic virus (CHV) (Johnson, 1984), was reported by Bang (1971) in a single European shore crab from Roscoff, France. A temporary reduction in the number of circulating haemocytes and reduced blood coagulation occurred 2–19 days post-infection in 74% of shore crabs inoculated with infected haemolymph (Bang, 1971). Recovery of coagulative properties occurred in 50–60% of the crabs 4–6 days later. The remaining crabs died (Bang, 1974). CHV causes haemocyte aggregation in the connective tissues of the hepatopancreas, gills, heart and ovary and is associated with extensive displacement of ovarian tissue, focal haemocyte necrosis and striated muscle damage (Hoover and Bang, 1978). The impact of CHV on wild populations of shore crabs is unknown (Brock and Lightner, 1990).

CHV virions are spherical, 55–80 nm in diameter and associate with the Golgi apparatus. This, along with the apparent haemagglutinating properties of the virus, forms the basis for the tentative association with the Bunyaviridae; however, no enveloped particles have been detected and the nucleic acid composition has yet to be determined (Hoover and Bang, 1976, 1978).

Viruses of uncertain affinities

S-virus, originally reported in portunid crabs from the Mediterranean coast of France by Bonami and Vago (1971), has been tentatively designated Bunyaviridae-like by Johnson (1984). Bonami *et al.* (1971, 1975) also attribute Rhabdoviridae and Paramyxoviridae characteristics to the virus. Infections were found in weak and dying wild crabs, as well as healthy individuals (Bonami *et al.*, 1971, 1975; Bergoin *et al.*, 1982); however, the precise correlation between clinical signs and infection is unclear. Experimental inoculations with infected tissue homogenates and purified virus resulted in 70–80% mortality within 20 days.

S-virus infects cardiac tissues and the endothelial cells of hepatopancreatic blood vessels (Bonami and Vago, 1971). Virions are enveloped, ovoid (80–130×190–130 nm), elongate (50–70×240–320 nm) or spherical (80–150 nm in diameter) and have surface projections. The nucleic acid is believed to be single-strand RNA (Bonami *et al.*, 1975; Bergoin *et al.*, 1982) and replication is cytoplasmic. S-virions bud into intracytoplasmic vesicles, from which they are released into intercellular spaces (Bonami *et al.*, 1975).

A virus resembling S-virus was reported from intracytoplasmic vesicles of the Y-organ of the Mediterranean shore crab (Zerbib *et al.*, 1975); however, no additional information on this virus has been produced since the original report.

Viruses of Nephropidae Dana, 1852 (genus *Cherax*)

There are two reports of a non-occluded Baculoviridae in Australian redclaw crayfish (*Cherax quadricarinatus*). The first described infections of the hepatopancreas in farmed redclaw crayfish from Australia (Anderson and Prior, 1992) may be related to a subsequent report of a similar virus in the same species being farmed in California (Groff *et al.*, 1993). The virus does not appear to cause mortality, although farmed redclaw crayfish in Australia showed poorer growth than uninfected wild populations. Both reports describe hypertrophied nuclei of infected hepatopancreas tubules containing loosely enveloped, rod-shaped virions, 292×102 nm (nucleocapsid: 216×47 nm).

Bacterial Infections

Bacteria of Bivalvia Linné, 1758

An excellent overview of bacterial diseases of bivalve molluscs is provided by Paillard *et al.* (2004), including specific analysis of knowledge to date and immunological/genetic research trends enhancing our understanding of several of the more significant diseases described below.

Rickettsia-, chlamydia- and mycoplasma-like organisms

Proteobacteria, Class Alphaproteobacteria, include the Family Rickettsiaceae comprising, *inter alia*, the orders Rickettsiales, Chlamydiales and Mycoplasmatales. These intracellular bacteria infect a wide range of bivalves and crustaceans. With certain exceptions, described below, Rickettsiaceae are Gram-negative, intracytoplasmic, membrane bound, oval to rod-shaped bacilli, measuring 0.3–0.6×0.8–2.0 μm. Chlamydiales are also Gram-negative, but have a complex developmental cycle, multiplying within cytoplasmic vacuoles and producing small, rigid-walled, infectious stages (elementary bodies). Larger, non-infectious forms (reticulate or initial bodies) have flexible walls and divide by fission producing intermediate or ellipsoid bodies. Chlamydias are coccoid or pleomorphic and generally measure 0.2–1.5 μm in diameter. *Mycoplasma*-like organisms (MLOs) are associated tentatively with the Mollicutes: small (0.3–0.8 μm), wall-less, prokaryotes which vary in shape from spherical to filamentous.

Generally, rickettsias, chlamydias and MLOs cause negligible tissue pathology (Harshbarger *et al.*, 1977; Comps *et al.*, 1979; Meyers, 1979b; Comps, 1982, 1983; Lauckner, 1983; Elston, 1986a) (Fig. 20.10a–d). Some significant exceptions, however, have been reported (Elston, 1986b; Olson and Pierce, 1988; Leibovitz, 1989; Bower and Meyer, 1991). Gill rickettsias of adult giant sea scallop (*Placopecten magellanicus*) in New England were associated with myodegeneration and mass mortalities (Gulka *et al.*, 1983; Gulka and Chang, 1984a,b); however, similar infections in other giant sea scallops have shown no associated histopathology or mortalities (Morrison and Shum, 1983; McGladdery, 1990; Getchell, 1991; Karlsson, 1991; McGladdery *et al.*, 1993; Ball and McGladdery, 2001). Rickettsial-like organisms (RLOs) in the gills of adult St Jacques scallops (coquille St Jacques; *P. maximus*) in France were also linked to mortalities (Le Gall *et al.*, 1988, 1991). Infected gill epithelia showed cytoplasmic degeneration, but no myodegeneration. In contrast, RLOs in the gills of Japanese scallops (*P. yessoensis*) are not associated with pathology (Elston, 1986b; Friedman, 1994). Similar, apparently benign, lesions were associated with rickettsial infections of epithelia in the Peruvian calico scallop (*Argopecten purpuratus*) from Chile (Lohrmann *et al.*, 2002).

Infections of various clam species are also rarely linked to pathology (Carballal *et al.*, 2001; MacCallum *et al.*, 2001; Marshall *et al.*, 2003). One exception, however, is a report of high mortalities in 6- to 9-month-old cultured bear-claw/horse-hoof clams (*Hippopus hippopus*) in the Philippines and Malaysia. The mortalities were associated with heavy gill infections by RLOs (Norton *et al.*, 1993). The precise factors triggering pathogenic infections are unknown, but, as with other bacterial infections of shellfish, adverse environmental conditions (overcrowding, limited water exchange) and physiological stress (transportation and handling) are believed to have played a role (Morrison and Shum, 1983; Leibovitz, 1989).

Nuclear inclusion X (NIX) causes sporadic late summer and early autumn mortalities in Pacific razor clams (*Siliqua patula*) from the north-western USA and British Columbia (Elston, 1986a; Olson and Pierce, 1988). NIX is unlike most rickettsias, infections being intranucleic rather than cytoplasmic. The only other endonucleic bacterium described to date occurs in the gill epithelium of the carpet clam (*Tapes decussatus*) (Azevedo, 1989). NIX is also abnormally large (16×25 μm) for an intracellular prokaryote, and the hypertrophied

host cells are visible to the naked eye as nodules in the gill filaments.

Larval bivalves are particularly susceptible to lethal infections by intracellular prokaryotes (Leibovitz, 1989; Bower and Meyer, 1991). The first epizootic attributed to chlamydial infection was in larval bay scallop (*A. irradians*) at two eastern USA

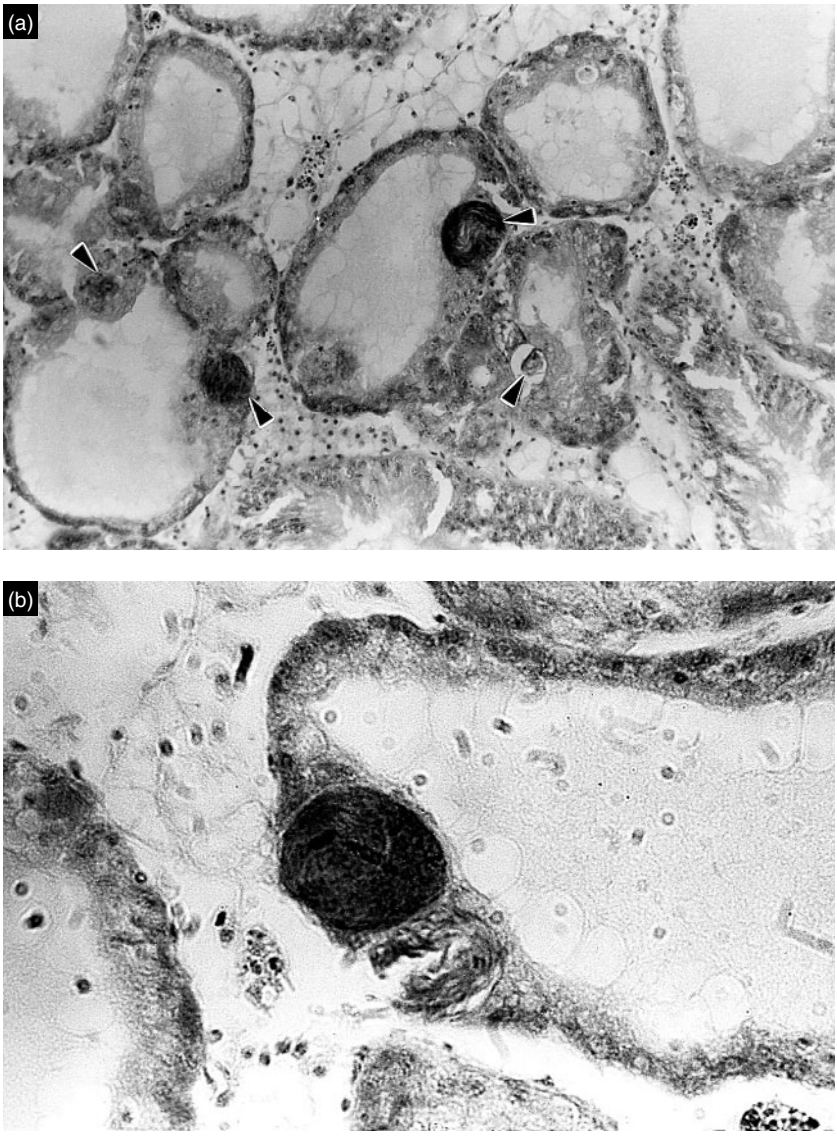


Fig. 20.10. (a) Chlamydial-like inclusion bodies (arrows) in the digestive tubules of *Crassostrea virginica* (H & E, ×160). (b) Different developmental stages of a filamentous chlamydial-like infection of the digestive tubules of *Crassostrea virginica*. Note rupture of the basal membrane, rather than the apical membrane, by one colony (H & E, ×630).

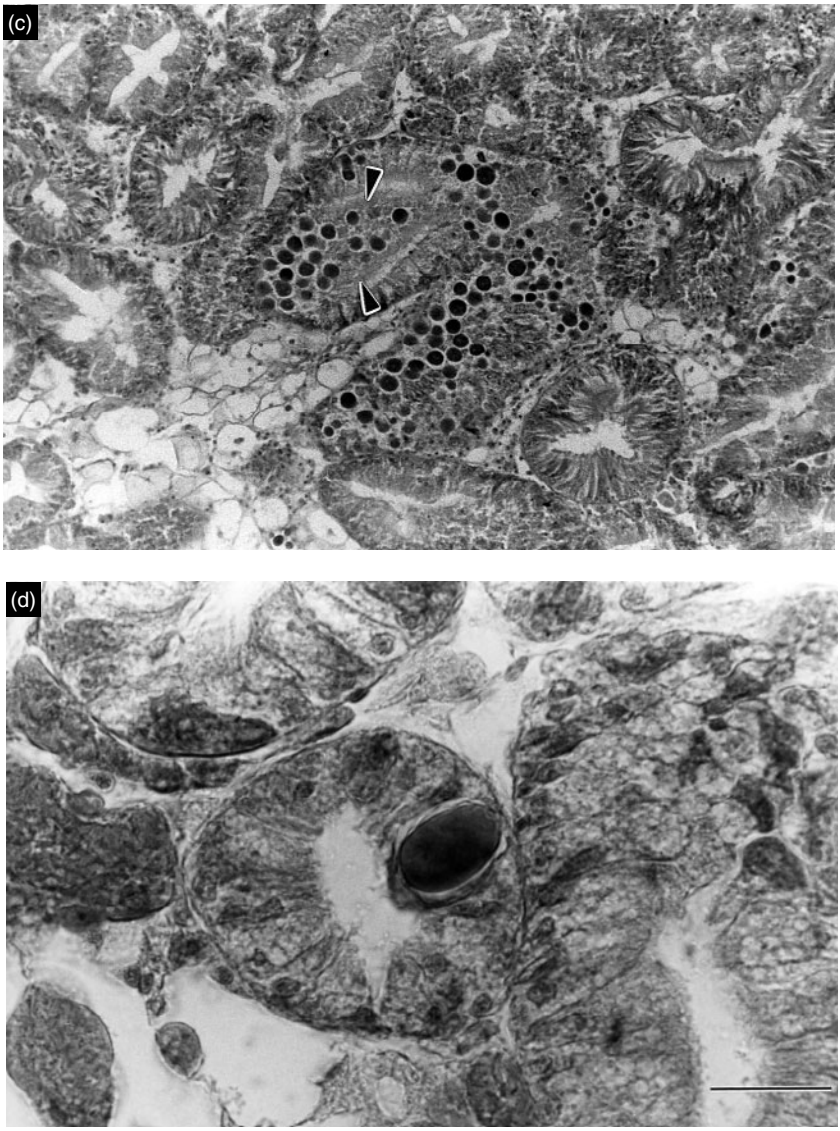


Fig. 20.10. (c) Heavy tubule epithelium infection by rickettsial-like organisms (dense spheres) in *Crassostrea virginica*. Note massive hypertrophy of some infected cells (arrows) (H & E, ×160). (d) Rickettsial-like colony in a tubule epithelial cell of the mussel *Mytilus edulis* (H & E, ×250).

shellfish hatcheries (Leibovitz, 1989). Basophilic inclusion bodies were found in disrupted digestive duct and tubule epithelia. Similar inclusion bodies infect adult bay scallops, but are not associated with disease (Morrison and Shum, 1982, 1983; Getchell, 1991; Karlsson, 1991). High stocking densities and water temperatures (>20°C) may have enhanced microbial proliferation during larval metamorphosis.

'Intracellular bacterium disease' produces pinkish-orange pustules in the adductor muscle of young Japanese scallops and was associated with poor growth and high mortalities at several grow-out sites in British Columbia (Bower and Meyer, 1991).

Experimental inoculations with high concentrations of lesion homogenate caused mortalities 2–4 weeks post-injection. Lower concentrations resulted in a 2 month prepatent period and development of adductor muscle lesions. Intracellular prokaryotes, tentatively identified as *Mycoplasma*-like, were found in some of the haemocytes within the abscess lesions (Bower and Meyer, 1991, 1994; Bower *et al.*, 1992, 1994a) (Fig. 20.11a,b).

Another MLO was described from edible cockles (*C. edule*) in central Portugal

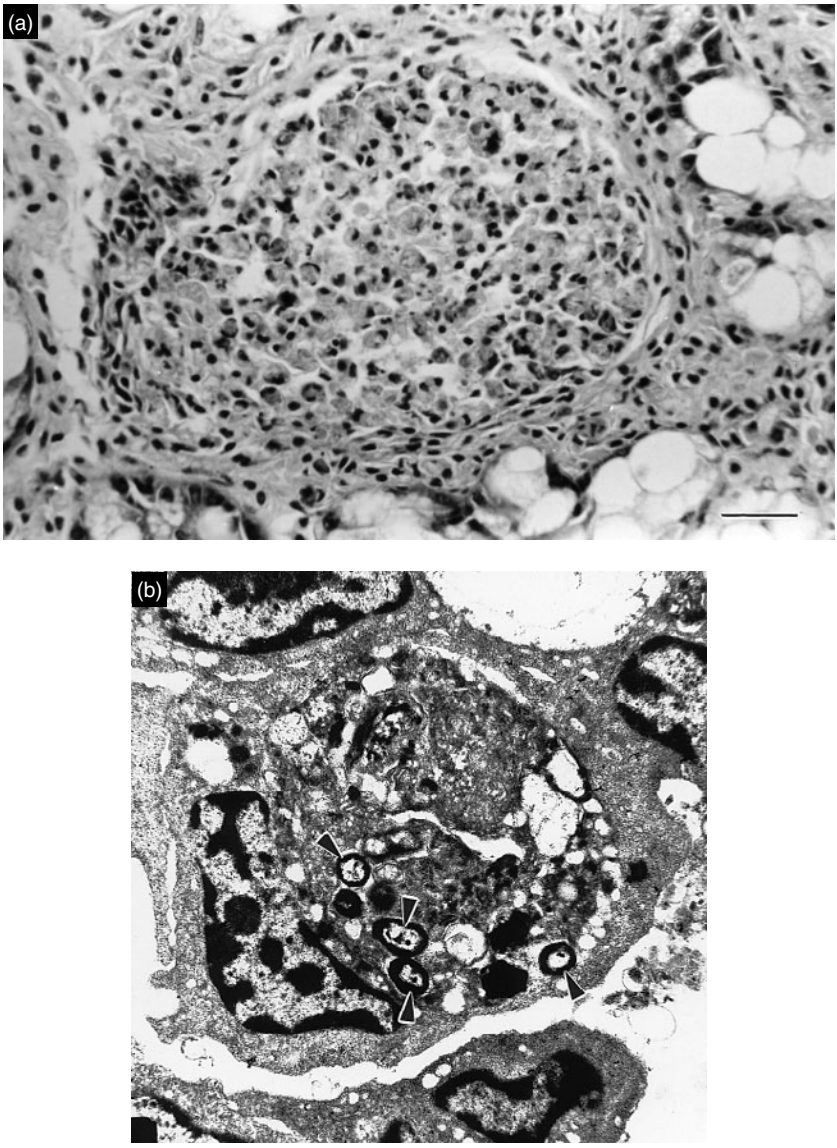


Fig. 20.11. (a) Encapsulated lesion of inflammatory haemocytes in the connective tissue of the digestive gland of *Patinopecten yessoensis* associated with infection by an intracellular bacterium. Photograph courtesy of S.M. Bower (H & E, scale bar=25 μm). (b) Electron micrograph of a haemocyte of *Patinopecten yessoensis* containing bacteria-like organisms (arrows). Infected haemocytes occur on the periphery of encapsulated inflammatory lesions (Fig. 20.11a). Photograph courtesy of S.M. Bower.

(Azevedo, 1993) which were suffering high summer mortalities. Infected gill epithelia demonstrated pycnotic nuclei, reduced mitochondria and no ribosomes. The MLOs have a trilaminar membrane, measure 0.5–4.0×0.9μm, lack a cell wall and are located in the cytoplasmic cisternae of the basal portion of infected cells. Attempts to transmit the infection under laboratory conditions have failed and the pathogenic significance in wild cockle populations is unknown (Azevedo, 1993).

Gram-positive bacteria

Relatively few Gram-positive bacterial diseases have been described from bivalves; however, *Nocardia* sp. is a Gram-positive, acid-fast Actinomycete, responsible for the disease Pacific oyster nocardiosis (PON) infecting Pacific oysters along the west coast of the USA, British Columbia and Japan (Imai *et al.*, 1968; Elston *et al.*, 1987; Friedman *et al.*, 1988, 1991; Friedman and Hedrick, 1991). Mortalities of up to 35% were reported in late summer and autumn. Yellow-green pustules, up to 1cm in diameter, in the mantle, gills, adductor and cardiac muscle were associated with intense haemocyte infiltration around the branching mycelial colonies of *Nocardia* (which distinguishes them from other basophilic, PAS-positive bacteria) (Friedman *et al.*, 1991) (Fig. 20.12).

Gram-positive bacteria have also been isolated from abscess lesions in giant sea scallops (*P. magellanicus*) from Atlantic USA and Canada (Sherburne and Bean, 1986; Getchell, 1991; McGladdery *et al.*, 1993). The lesions are visible as 'brown spots', up to 3mm in diameter, on the adductor muscle, which renders the meat unmarketable. 'Bacterial abscess disease' (or 'brown spot disease') has a patchy distribution in lower salinity estuarine environments. Prevalences are generally less than 4%, although some foci may reach 25%. Larger scallops appear most severely affected, but there is no direct correlation to shell fouling or population density. Histologically, the disease is similar to intracellular bacterium disease of Japanese scallops (mycoplasma disease described above) with systemic focal lesions containing Gram-

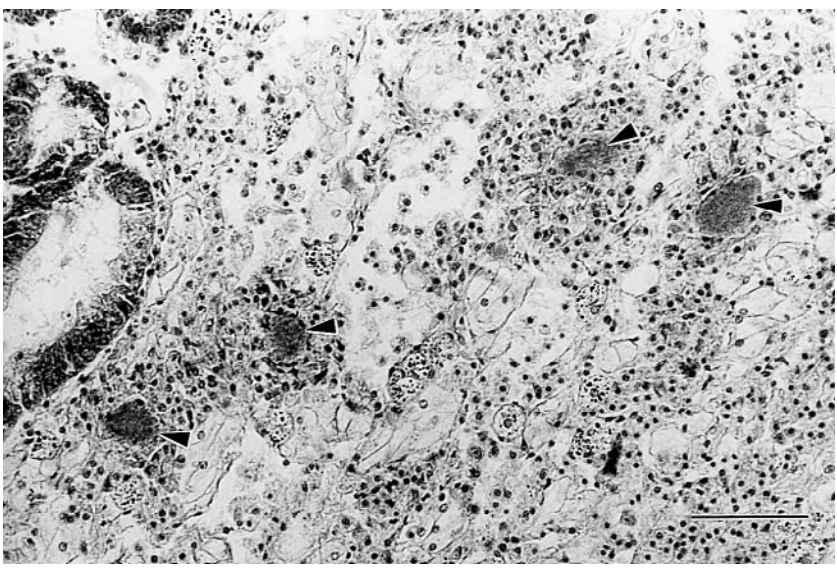


Fig. 20.12. Aggregations of Gram-positive actinomycete *Nocardia* bacteria in the connective tissue of *Crassostrea gigas* (arrows). Note infiltration of haemocytes around the bacteria (H & E,×160).

positive, pleomorphic, bacteria (Fig. 20.13). Attempts to culture and identify the 'brown spot disease' bacteria have been unsuccessful to date (Getchell, 1991). Gram-positive bacteria (*Micrococcus* sp.) have been observed in abscess lesions from Canada and the USA (Getchell, 1991), but Gram-negative species (*Vibrio*, *Pseudomonas* and *Aeromonas*) have also been cultured. All identified genera are ubiquitous, mainly aerobic bacteria, while systemic infections suggest an anaerobe involvement. This may indicate a non-pathogen-specific aetiology or difficulty in preventing contamination by surface or secondary histolytic bacteria.

Gram-negative bacteria

A number of disease problems in bivalve hatcheries are attributed to Gram-negative bacteria (Walne, 1958; Tubiash *et al.*, 1965, 1970; Elston *et al.*, 1981, 1982; Lodeiros *et al.*, 1987; Dungan and Elston, 1988; Dungan *et al.*, 1989; Elston, 1989; Nicolas *et al.*, 1992b), especially *Vibrio* spp.. Although some have been identified to species (*V. anguillarum*, *V. alginolyticus* and *V. tubiashii*), most are identified only to genus (Hada *et al.*, 1984). Severity of infection is related to suboptimal growing conditions (e.g. accumulation of dead or dying larvae, contaminated algal food, residual gametes) which enhance bacterial proliferation and compromise the immune responses of infected larvae (Elston, 1989); however, sensitivity to *Vibrio* spp. can vary considerably. Sindermann (1988a) cites 10^2 *Vibrio* cells/ml as potentially pathogenic to Pacific oyster larvae, while other bivalves may tolerate 10^5 cells/ml (Perkins, 1993). Thus, a generalization of potentially pathogenic concentrations of *Vibrio* is not possible and threshold tolerances need to be established for individual holding systems, bivalve species and seasonal cycles of production.

Vibrio spp. produce exotoxins (ciliostatic factors and haemolysins) which cause tissue necrosis in bivalve larvae (Brown and Roland, 1984; Nottage and Birkbeck, 1986; Nottage *et al.*, 1989). High water temperature and excessive stocking densities increase the severity of infection (Elston, 1989), especially in pre-metamorphic bivalves (although

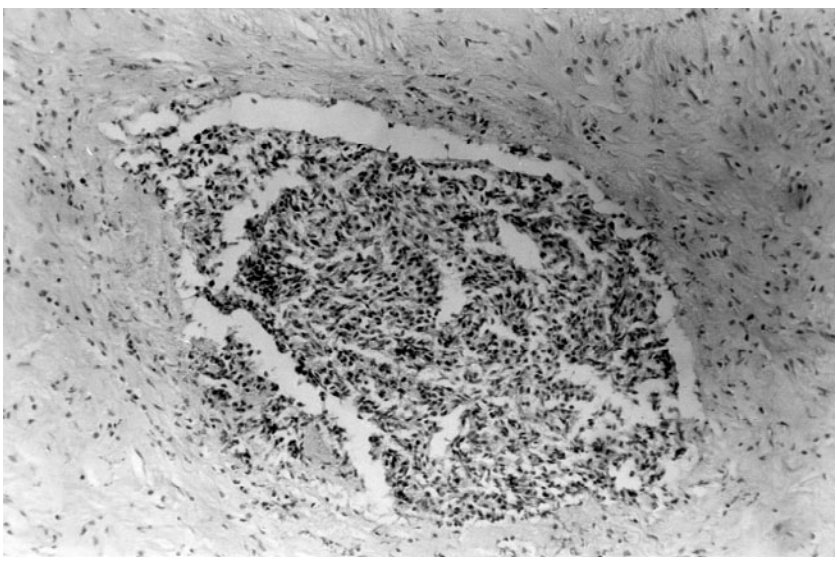


Fig. 20.13. Bacterial abscess lesion in the adductor muscle of *Placopecten magellanicus*. Note focal aggregation of necrotic haemocytes and myodegeneration (H & E, ×25).

postmetamorphic juveniles are also infected), hence the name 'larval necrosis' or 'bacillary necrosis' (Tubiash *et al.*, 1965). *V. anguillarum* isolated from the pericardial fluid of adult oysters with cardiac oedema was found to be highly pathogenic to larval oysters infected experimentally (Tubiash *et al.*, 1973); however, pathogenic infections in adult bivalves are rare. *V. anguillarum* and *V. tubiashii* show minimal host specificity and affect a wide range of molluscan species (Brown, 1981; Jeffries, 1982; Garland *et al.*, 1983; Lodeiros *et al.*, 1987).

One exception to pathogenicity in adult bivalves is *V. tapetis*, initially called *Vibrio* predominant 1 (*Vibrio* P1 group or strain) (Allam *et al.*, 1996), which is the cause of brown ring disease (BRD) in Manila (*Ruditapes philippinarum*) and carpet (*R. decussatus*) clams along the Atlantic coasts of Europe (Maes and Paillard, 1990; Paillard and Maes, 1994; Paillard *et al.*, 1994) and, less frequently, in the Mediterranean and Adriatic seas (Paillard, 2004). It is a fermentative, Gram-negative, motile, non-sporulating curved rod that is oxidase-positive and growth inhibited by vibriostat O/129. It is differentiated from other species of *Vibrio* by growth at 4°C; no growth above 22°C or at salinities >5% NaCl; no production of arginine dehydrolase, lysine decarboxylase or ornithine decarboxylase; and a negative Voges–Proskauer reaction (Borrego *et al.*, 1996).

Manila clams appear most susceptible to mortalities associated with BRD, although it has also been isolated from gold carpet shell (*Venerupis aurea*) and edible cockles from Quiberon, France, with no evidence of disease. It has also been transmitted experimentally to the banded carpet shell (*T. rhomboids*). *V. tapetis*-like strains have also been reported from cultured fish (corkwing wrasse, *Crenilabrus* (= *Symphodus*) *melops*), during clam mortality events in Norway (Jensen *et al.*, 2003), as well as in halibut (*Hippoglossus hippoglossus*) in Scotland (Paillard, 2004).

In 2006, *V. tapetis* was reported from Manila clams on the west coast of Korea. Although losses in clam production were linked primarily with high levels of *Perkinsus olseni* (a protistan disease of clams), the involvement of other pathogens such as *V. tapetis* was investigated (Park *et al.*, 2006). A positive correlation between a higher parasite load and signs of BRD was found, which is similar to observations of infections of Manila clams from areas of southern Europe (Figueras *et al.*, 1996).

V. tapetis adheres to and progressively colonizes the surface of the periostracal lamina at the mantle edge of the shell. This causes abnormal deposition of periostracum around the inner shell surface, resulting in accumulation of the brown organic material symptomatic of the disease. Infection also disturbs the calcification process of shell deposition and growth. Experimental inoculations induced clinical expression of the disease within 12 h to 4 weeks post-infection (Oubella *et al.*, 1994). Experimental infections using other strains of *Vibrio* failed to elicit similar thickening of the periostracum. Nutritional stress is also thought to be a possible contributing factor in the pathogenicity of BRD infections (Plana and Le Pennec, 1991).

Allam *et al.* (2002) concluded that clam mortality associated with BRD likely resulted from the penetration of *V. tapetis* into the extrapallial space through disruption of the periostracal lamina. The bacteria eventually penetrate the mantle epithelium and soft tissues, where proliferation causes severe damage and subsequent death.

Infected clams showed a significant decrease in glycogen, suggesting a degeneration of metabolic activity (Plana *et al.*, 1996). A negative correlation between the level of BRD infection and the condition index of clams (ratio of soft tissue wet weight to shell dry weight) was also noted by Park *et al.* (2006) in Korea.

Allam *et al.* (2001) demonstrated that Manila clams from southern Puget Sound, Washington, USA, were more resistant to BRD than the same species of clam from the Bay of Brest, Brittany, France, and the carpet clam from the Bay of Brest was more resistant than both stocks of Manila clam. Their results suggested that resistance to BRD might be related to the concentration of granular haemocytes and the phagocytic

activity of the haemocytes. Several additional studies of Manila clam defence responses to *V. tapetis* and other *Vibrio* spp., compared with those of other bivalves (Allam and Paillard, 1998; Allam *et al.*, 2000a, 2000b, 2006; Choquet *et al.*, 2003; Reid *et al.*, 2003; Allam and Ford, 2006), support this hypothesis.

An apparently similar 'brown ring disease' appeared in the late 1980s in juvenile American oysters in north-eastern USA (Bricelj *et al.*, 1992; Davis and Barber, 1994). Following studies into various putative aetiologies, including *Vibrio* spp. (due to the clinical similarities with BRD of Manila clams), 'juvenile oyster disease' (JOD) has been attributed recently to heavy colonization by the novel species *Roseovarius crassostreae* (Maloy *et al.*, 2007). This led authors to re-designate the disease name as ROD (Roseovarius oyster disease). Clinical signs of ROD are reduced growth rates, fragile and uneven shell development, cupping of the left valve, mantle retraction and deposition of a conchiolin ring around the inner growth edge of the shell (Bricelj *et al.*, 1992; Davis and Barber, 1994). These signs appear 4–6 weeks after the transfer of seed from the hatchery to grow-out (nursery) sites, and precede mortality events of 50 to >90% of total production in a single week (Barber *et al.*, 1996).

Initial suspicion of non-*Vibrio* bacterial involvement was pursued following work on two antibacterial antibiotics (norfloxacin and sulfadimethoxine-ormetoprim), which were used to treat cultured juvenile oysters (Boettcher *et al.*, 1999). The antibiotics delayed the onset of juvenile mortalities and reduced mortality rates significantly compared with controls. Subsequent investigation revealed a previously undescribed species of the marine α-proteobacteria, Roseobacter group. These bacteria were recovered at low levels, or not at all, from oysters that survived the ROD epizootic.

Another *Vibrio* species from Manila clams (VTP – *Vibrio* of *T. philippinarum*) is more pathogenic to larvae (Nicolas *et al.*, 1992b) than adults. As with *V. tapetis*, VTP shows unusual host specificity, being non-pathogenic to coquille St Jacques scallop and Pacific oysters. In addition, VTP has a short survival time (4–5 days, compared with 7 days for BRD) in seawater, is resistant to chloramphenicol and does not grow on TCBS agar. VTP-disease was eradicated successfully by drying out the affected hatchery and surrounding facilities and has not been reported since 1987 (Nicolas *et al.*, 1992b).

High mortalities (up to 80%) of gold-lipped pearl oysters (*P. maxima*) in north-western Australia have been linked to *Vibrio* infections (Dybdahl and Pass, 1985; Pass *et al.*, 1987). *V. harveyi* isolated from the haemolymph of infected pearl oysters were used to induce the same clinical signs seen in wild infections, including anamolous conchiolin deposits and disruption of nacre blister formation. The disease was linked to holding conditions during transportation from wild beds to a pearl oyster farm, especially when water temperatures were low (18°C). Mortalities were reduced successfully by avoiding transportation during the Austral winter, reducing stocking densities and shortening the shipment time (Pass *et al.*, 1987).

Cytophaga-like bacteria (CBL), belonging to the gliding bacteria group (including *Flexibacter* spp.), cause hinge ligament disease in juvenile American, Pacific and European flat oysters; and hard-shell, Manila and razor (*S. patula*) clams (Elston, 1984; Dungan and Elston, 1988; Dungan *et al.*, 1989). Healthy bivalves appear capable of controlling the infection, whereas individuals compromised by adverse growing conditions, or physiological stresses (as described for vibriosis above), appear more susceptible (Elston *et al.*, 1982; Elston, 1984). Infections generally affect juveniles <1 cm in length, causing liquefaction of the hinge ligament (especially under warm conditions, 10–20°C) and impeding feeding and respiration. Hinge ligament disease also increases secondary bacterial and fungal infections. CLBs are distinguished from other bacteria by motility, variable size range (2.5 to >200 μm long) and slow growth on cytophaga or flexibacter agar (see the section on culture of microorganisms).

Various species of filamentous cyanobacteria including *Plectonema terebrans*,

Hyella caespitosa, *Mastigocoleus testarum*, *Mastigocoleus* sp. (Nostochopsidaceae) and aggregated (pseudofilamentous) cyanobacterium *Pleurocapsa* sp. have been associated with shell damage in various mussel species cultured along the western Cape of South Africa, from Saldanha Bay to Simonstown and the Bathurst and Peddie districts. Similar infestations of mussel shells have also been reported from Sierra Leone. Gallo mussels (*M. galloprovincialis*), introduced to South Africa around the 1970s for culture purposes, appear particularly vulnerable, although brown mussel (*P. perna*) and other native species of mussels (black mussel [*Choromytilus meridionalis*] and Magellan/ribbed mussel [*Aulacomya ater*]) are also affected. The endolithic cyanobacteria create numerous tiny burrows, compromising the mussel shell strength and leaving them vulnerable to predation and heavy wave action. In some cases, the bacterial tunnels lead to complete perforation of the shell and mortality. Recorded prevalences vary from 94% at high intertidal sites to 1% at subtidal sites. Webb and Korrûbel (1994) noted a correlation between heavy infestation and high population density, high tidal position and heavy wave action. Abrasion to the periostracum appears to predispose mussels to infection (Kaehler, 1999). Interestingly, although intertidally grown gallo mussels were severely affected, those cultured on hanging lines in the same area were virtually free of infection. The native Magellan mussel also appears to be susceptible to shell damage from these endolithic cyanobacteria (Webb and Korrûbel, 1994). All reports indicate that damage from the bacterial excavations is usually limited where environmental predisposing factors are also limited (i.e. erosion of the periostracum, high tidal and wave action). However, where shell damage was heavy, especially around the site of adductor muscle attachment, reproductive output was also reduced (Kaehler and McQuaid, 1999).

Bacteria of Cephalopoda Cuvier, 1797

Young octopuses held at high densities in recirculated seawater suffer severe bacterial ulceration (Hanlon *et al.*, 1984). Erosion of the mantle epidermis destroys the underlying chromatophores, leaving bare, white patches. Subsequent necrosis of the underlying musculature and internal organs leads to death within as few as 4 days.

Bacteria isolated from lesions on Atlantic pygmy octopus (*O. joubini*) and Caribbean reef octopus (*O. briareus*) were all Gram-negative: *V. alginolyticus*, *V. damsela*, *V. parahaemolyticus*, *Pseudomonas stutzeri* and *Aeromonas cavia*. A pseudomonad and *Acinetobacter anitratus* were also isolated from lesions on a single North Pacific giant octopus (*O. dofleini*) held at a public aquarium in the USA. After 2 months, the lesions began to proliferate, but healed completely when a tetracycline treatment was administered via its live prey (Stoskopf *et al.*, 1987, cited in Hanlon and Forsythe, 1990).

Laboratory reared two-spot octopus (*O. bimaculoides*) and Mexican four-eyed octopus (*O. maya*) dying hours after partial loss of one to two arms, were found to have soft sections in their intact limbs. Examination of these sections and the stumps of shed arms revealed *V. carchariae* (Hanlon and Forsythe, 1990). The source of infection was believed to be frozen shrimp. Water bath treatment with chloramphenicol was effective but post-treatments were required after 1–3 weeks (Hanlon and Forsythe, 1990) (see the section on chemotherapy, under treatment and protection).

A major cause of mortality in wild-caught squids is secondary bacterial infection of skin and fin lesions sustained during capture. Leibovitz *et al.* (1977) described necrotic dermatitis with deep penetration of the muscle tissue in captive long-finned squid (*Loligo pealei*). Although the exact aetiology was not determined, the consistent presence of Gram-negative filamentous bacteria, resembling *Myxobacteria* spp., was noted. *V. anguillarum* was also isolated from skin surfaces, along with an unidentified enterobacterium and two Gram-negative rods. This diversity of bacterial isolates suggests a secondary rather than primary role in proliferating lesions in

wild-caught squid (Ford *et al.*, 1986; Hanlon and Forsythe, 1990).

Eye infections commonly afflict captive squid following abrasion of the corneal surfaces (Hanlon and Forsythe, 1990). Bacteria proliferate in the aqueous humour and cause swelling, which can be fatal if the corneal surface is perforated. *Micrococcus* sp. was isolated from the aqueous humour of captive veined squid (*L. forbesi*). *V. harveyi* and *V. carchariae* were isolated from bigfin reef squid (*Sepioteuthis lessoniana*). There are no reports of eye infections in wild squid.

Hanlon and Forsythe (1990) also report a severe systemic infection of captive European cuttlefish (*S. officianalis*), which caused 90% mortality within 7 days of the appearance of swollen dorsal blood vessels. These developed into a haematoma which ruptured the dorsal epidermis. *V. splendidus*, *V. pelagius* (Biogroup II) and *P. stutzeri* were isolated from the haemolymph. Treatment with chloramphenicol or gentamycin prior to development of the dorsal haematoma was successful in halting the infection (Hanlon and Forsythe, 1990).

Bacteria of Gastropoda Cuvier, 1797

Juvenile vibriosis in red abalone (*H. rufescens*) from a hatchery in California (Elston and Lockwood, 1983) and northern/pinto abalone (*H. kamtschatkana*) from a hatchery in British Columbia (Bower *et al.*, 1994a) was responsible for swollen patches of epipodial tissue which became necrotic and ruptured. Small numbers of bacteria were detected on sloughed-off epithelium (Elston and Lockwood, 1983), as well as from the intramuscular vascular sinuses and along peripheral neurons. Nine isolates from the Californian hatchery and diseased abalones were related to the *Vibrio* group (Gram-negative, facultatively anaerobic, non-aerogenic, oxidase-positive rods with single polar flagella) (Elston and Lockwood, 1983).

Vibrio spp. including *V. harveyi*, *V. carchariae* (possible synonym of *V. harveyi*), *V. splendidus* I, *V. alginolyticus* and other bacteria (*Clostridium lituseberense*, *Flavobacterium*-like bacteria and long *Flexibacter*/*Cytophaga*-like rod bacteria) have been associated with juvenile abalone vibriosis disease losses. These do not include *V. fluvialis* II, the cause of blister disease in abalone in China, described below. Vibriosis has been reported from abalone culture facilities around the world, including California (USA), Baja California (Mexico), Australia, New Zealand, South Africa and British Columbia (Canada). Such infections have also been associated with mass mortalities of wild abalone on the northern coast of France (Bower, 2006).

Species affected include red abalone and northern/pinto abalone (S.M. Bower, unpublished records), black-lip abalone (*H. rubra*), green-lip abalone (*H. laevigata*), green ormer abalone (*H. tuberculata*), midas ear abalone (*H. midae*) and paua shell abalone (*H. iris*). Systemic infections cause tissue necrosis and death, but can be managed in culture situations over the short larval period of development using stringent sanitary practices.

In Tasmania, Australia, outbreaks in cultured black-lip, green-lip abalone and their hybrids were associated with two species of *Vibrio* (*V. harveyi* and *V. splendidus* I) and *Flavobacterium*-like bacterium. In most cases, stress factors (e.g. high temperatures, grading trauma, anaesthetics, gradual increase in salinity in the recirculation system, etc.) were reported to have precipitated the diseases (Handlinger *et al.*, 2001, 2002, 2005).

In Kanagawa Prefecture, Japan, *V. carchariae* (possible synonym of *V. harveyi*) was associated with mass mortalities of Japanese abalone (*H.* [= *Sulculus*] *diversicolor supratexta*). Infections were characterized by white spots on the abalone foot, consisting of necrotic muscle fibres and bacteria and high mortalities (Nishimori *et al.*, 1998). *V. carchariae* was also identified as the probable cause of mass mortalities of green ormer abalone in the natural environment along the Brittany and Normandy coasts of France and in a land-based abalone farm in Normandy (Nicolas *et al.*, 2002).

Another disease of cultured abalone, known as 'blister disease' has been described

recently from facilities in the Dalian region of China (Guo *et al.*, 1999; Nie and Wang, 2004). Mortalities of up to 50–60% have been reported from cultured Japanese disc abalone (*H. discus hannai*). *V. fluvialis* II was isolated from blister-like lesions and induced identical lesions using challenge experiments. Initial lesions were observed in the surface tissues of the foot, from where they expanded into the inner tissues as the disease progressed. Three strains of *V. fluvialis* II were isolated and demonstrated broad spectrum antibiotic resistance related to gene mutations (Li *et al.*, 1996). Although the disease appears restricted to this area, *V. fluvialis* is commonly found in estuarine environments worldwide (Lee *et al.*, 1981).

In New Zealand, paua shell cultured at high densities showed lesions and exfoliation of the foot epithelium and epipodium. These were also associated with infections of various Gram-negative bacteria (Diggles and Oliver, 2005).

In addition to hatchery-reared abalone mortalities, one other bacterial disease responsible for serious losses of wild abalone is withering foot syndrome of abalone, also known as 'withering disease', 'foot withering syndrome' and 'abalone wasting disease'. It is caused by a new species and genus, *Xenohaliotis californiensis*, within the Class Proteobacteria, Order Rickettsiales, Family Anaplasmataceae (Friedman *et al.*, 2000; Dumler *et al.*, 2001), and appears closely related to the bacterial genera *Ehrlichia*, *Anaplasma* and *Cowdria*. Two forms of the bacterium are found: a bacillus form, measuring 332–1550 nm; and a spherical form, measuring 1450 nm in diameter. These reproduce via binary fission in intracytoplasmic vacuoles, 14–56 microns in diameter, found in the gastroepithelium (Gardner *et al.*, 1995; Friedman *et al.*, 2000).

It was first detected in mortalities in Diablo Cove, California, in the early 1990s, where disease and mortalities appeared limited to the vicinity of a warmwater discharge. In 1996, however, it spread northwards to the San Francisco area (Alstatt *et al.*, 1996; Finley and Friedman, 2000). High prevalences of *X. californiensis* were found in clinical and subclinical cultured and wild populations of red abalone, green abalone and pink abalone (*H. corrugata*) (Cáceres-Martínez *et al.*, 2000; Cáceres-Martínez and Tinoco-Orta, 2001; Álvarez-Tinajero *et al.*, 2002). Natural infections have also been found in black-lip, white abalone (*H. sorenseni*) and Japanese abalone (Wetchateng, 2008). Laboratory challenges have also produced infections in flat abalone (*H. wallalensis*) and Japanese disc abalone.

The disease appears most evident in black-lip abalone, although the other species may also show serious infections of the digestive gland tissues. Clinical signs include lethargy, retracted visceral tissues and atrophy of the foot muscle in all sizes of abalone. At 18–20°C, death usually occurs within 1 month of the appearance of the disease. Infected abalone show reduced kelp consumption, along with metabolic changes (lower oxygen consumption and greater ammonia excretion than unaffected abalone) (Kismohandaka *et al.*, 1993) before atrophy of the foot becomes evident (Friedman, 1996; Shields *et al.*, 1996; Friedman *et al.*, 1997a). Haemocyte defence responses also appeared to be compromised (Friedman *et al.*, 1999, 2000). Kismohandaka *et al.* (1995) observed foot muscle fibre depletion in advanced infections, although no pathogens were present, and suggested that abalone might have been using protein from the foot muscle as an alternative energy source.

Spread of the disease caused crashes in black-lip abalone populations on six of the eight California Channel Islands by 1992 (95–100% losses) and closure of the black abalone fishery in 1993. Dramatic increases in infection of cultured *H. rufescens* were also associated with elevated seawater temperatures related to El Niño (Moore *et al.*, 1999); however, survivors appeared to develop resistance/tolerance to further infections (Friedman *et al.*, 2003).

X. californiensis has been detected subsequently in cultured red abalone in Iceland and green ormer in Ireland in 2006, with no associated mortalities in both cases. These detections were reported to the OIE[6] and affected stocks were destroyed.

Bacteria of Nephropidae Dana, 1852 (genus *Homarus* Weber, 1795)

Gram-positive bacteria

One of the best-studied bacterial diseases of marine invertebrates is gaffkemia (or 'red-tail disease') of American lobster (*Homarus americanus*) and European lobster (*H. gammarus*) (see reviews by Stewart, 1980, 1984, 1993; Brock and Lightner, 1990; Sindermann, 1990). Gaffkemia is caused by *Aerococcus viridans* var. *homari* (syn. *Gaffkya homari* and *Pedicoccus homari*), a Gram-positive, tetrad-forming, non-motile, catalase-negative, β-haemolytic, facultative anaerobic coccus (Fig. 20.14a,b). The characteristic tetrad formations measure 0.8–1.1 µm (Hitchner and Snieszko, 1947, cited in Brock and Lightner, 1990).

Gaffkemia primarily causes problems in lobsters held in live-holding pounds. Wild lobsters and other decapod crustaceans act as reservoirs of infection (Stewart *et al.*, 1966; Stewart and Rabin, 1970; Vachon *et al.*, 1981), along with free-living stages of *A. viridans* var. *homari* in mud sediments and on lobster-holding pound surfaces. Experimentally, crabs (Jonah crab [*Cancer borealis*], Dungeness crab [*C. magister*], blue crab [*C. maenas*], belanger/portly spider crab [*L. emariginata*], snow crab [*Chionoecetes opilio*], red deep-sea crab [*Geryon quinquedens*], Atlantic rock crab [*C. irroratus*], spiny lobster [*Panulirus interruptus*]) and prawns (spot prawn [*Pandalus platyceros*] and brown shrimp [*P. aztecus*]) show negligible pathogenic effects, except after prolonged periods (Stewart, 1980). However, even low concentrations of the bacteria isolated 3 months post-infection from red deep-sea crab, Atlantic rock crab and snow crab were still highly virulent to American lobster (Stewart, 1980).

Abrasion of the epicuticle facilitates access to the lobster haemolymph by the bacteria, which causes a pinkish discoloration responsible for the name 'red-tail disease' (however, this is not specific to gaffkemia). The bacteria multiply and within 48 h spread to the skeletal muscle. By day 8 at 15°C, the number of circulating haemocytes decreases to almost zero and death occurs within 2 weeks. Mortality rate is linked to the condition of the lobster at the time of infection and to water temperature, but the precise mechanism by which the bacteria evade the lobster's antimicrobial defence systems is unknown (Stewart, 1980, 1993). Careful management of stocking densities, stringent monitoring of bacteria and lobster health, plus prompt removal of moribund lobsters, have reduced losses to gaffkemia significantly (Fisher *et al.*, 1978; Stewart, 1980).

Gram-negative bacteria

Vibriosis in lobsters has been reviewed by Rosemark and Fisher (1988). All mortalities occurred in captive juveniles and pathogenicity was related to suboptimal holding conditions. *V. anguillarum* and *Vibrio* sp. (BML 79-078) were isolated from the haemolymph of lobsters with vacuolized and oedematous hepatopancreas tissue. Experimental infections with 10^5 *Vibrio* spp. resulted in 66% mortality within 24 h (Bowser *et al.*, 1981); however, the effect of *Vibrio* spp. on wild lobster populations is unknown.

Gram-negative chitinolytic (= chitinoclastic) bacteria are common on both wild and captive crustaceans, including lobsters. Infections first appear as pits in the exoskeleton that may penetrate to the underlying epidermis, associated with erosion and melanization of the affected site. Common names applied to chitinolytic bacterial infections include shell disease, burn(ed) spot (= brandfleckenkrankheit), brown or black spot disease; however, these descriptive names also cover the clinical signs of chitinolytic fungal infections (see the section below on fungal infections). Recent studies have shown that chitinolytic bacteria account for a small fraction of the bacteria isolated from shell lesions (Christoserdov *et al.*, 2005); thus, 'bacterial shell disease', as used here, is not specific to chitinolytic bacterial infections.

As with gaffkemia, damage to the lipoprotein epicuticle facilitates access by the bacteria to the chitinous exocuticle and endocuticle (Stewart, 1980; Castro and

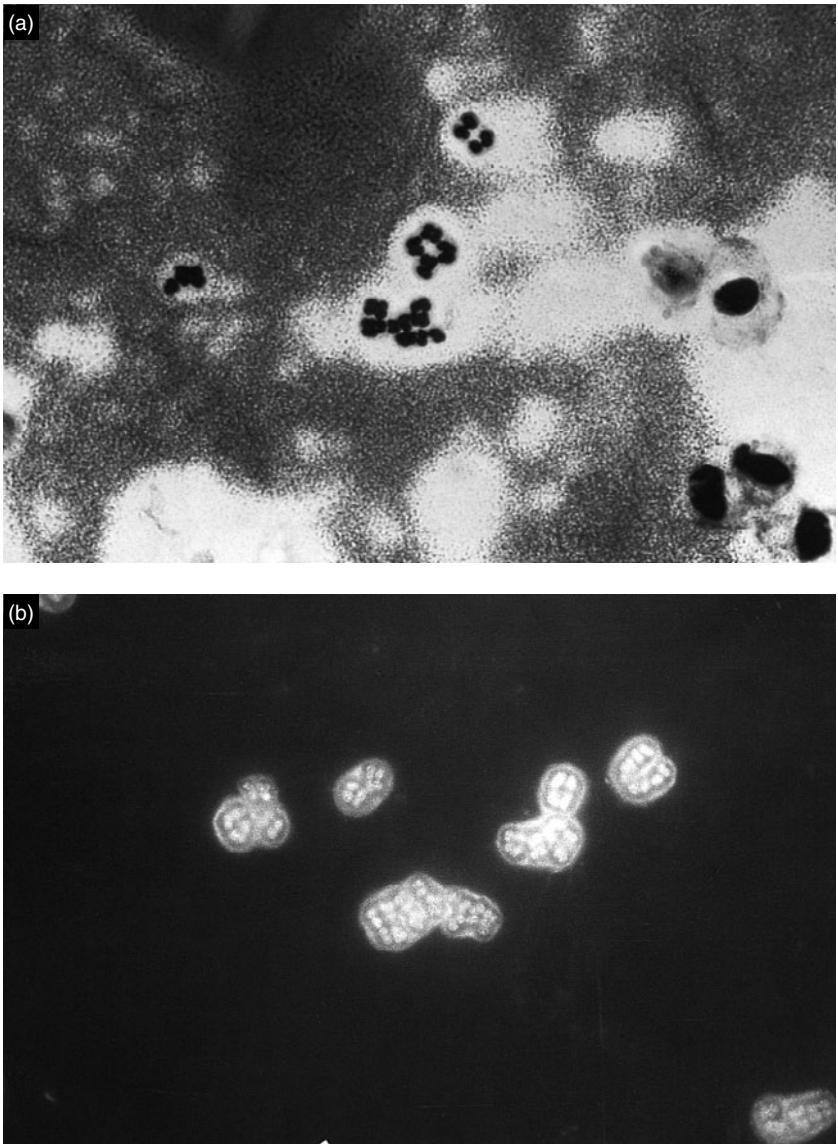


Fig. 20.14. (a) Gram stain of haemolymph containing *Aerococcus viridans* var. *homari*, the causative agent of gaffkemia in *Homarus americanus*. Note tetrad formation by individual cocci. Photograph courtesy of L. Marks and J.E. Stewart (Gram stain, ×1000). (b) Indirect immunofluorescent antibody stain of *Aerococcus viridans* var. *homari* (gaffkemia) in haemolymph from *Homarus americanus*. Photograph courtesy of L. Marks and J.E. Stewart (IFAT, ×1000).

Angell, 2000; Smolowitz *et al.*, 2005). Epicuticular damage is usually due to abrasion, but may also be caused by lipolytic microorganisms (Cipriani *et al.*, 1980). The chitin layers of the gills may also be infected, showing discoloration, thickening and, in

severe cases, complete destruction. Damage to the exocuticle is not generally a serious problem, except under stressful holding conditions, or when the cuticle is weakened after moulting (Fisher *et al.*, 1978; Stewart, 1984; Fisher, 1988a; Laufer *et al.*, 2005). Juvenile lobsters fed an inadequate diet showed greater epicuticle malformation and mortality due to bacterial shell disease than well-fed controls (Fisher *et al.*, 1978; Fisher, 1988a; Prince *et al.*, 1995). In addition, temperatures of 2–5°C impede epicuticle repair and enhance proliferation of *Vibrio* and *Pseudomonas* spp. (Malloy, 1978).

Infection of the exoskeleton tends to occur parallel to the shell and is defended against by melanization of the periphery of the lesion. This blocks proliferation of the bacteria and the infections can be shed during the next moult. If the melanization process develops adhesions to the subcuticular tissues, however, this can impede moulting and results in death (Sindermann, 1989).

Bacteria of Nephropidae Dana, 1852 (genera *Astacus*, *Camabrus*, *Cherax*, *Austropotamobius* and *Orconectes*)

Rickettsia-like organisms

The only rickettsial-like organism reported in crayfish infects Australian red-claw crayfish (*C. quadricarinatus*) from freshwater streams and culture facilities in northern Australia (Ketterer *et al.*, 1992). Mass mortalities are linked to basophilic, granular inclusions which cause hyperplasia and hypertrophy of endothelial and interstitial cells of the gills and other organs. The inclusions consist of thin-walled microcolonies containing rod-shaped, rickettsial-like organisms (0.16×0.5 μm). Poor water quality and excessive gill fouling were correlated positively to the severity of the disease (Ketterer *et al.*, 1992).

Gram-positive bacteria

A *Nocardia*-like (Actinomycete, acid-fast bacteria) infection reported from a single freshwater white-claw crayfish (*Austropotamobius pallipes*) in the UK (Alderman *et al.*, 1986; Alderman and Polglase, 1988) was associated with abnormal behaviour and melanized nodules throughout the tail musculature. Examination of the nodules revealed concentric layers of melanized haemocytes surrounding non-encapsulated foci of the acid-fast branching bacteria. Similar nodules have been associated with Gram-positive bacteria in white-leg shrimp (*P. vannamei*) (Lightner and Redman, 1986) and Malaysian/giant river prawn (*M. rosenbergii*) (Brock *et al.*, 1986a), although these infections were identified tentatively as *Mycobacterium*-like due to the rod-shaped bacilli. The effect of these types of bacterial infections on wild populations is unknown.

Gram-negative bacteria

Proteus vulgaris, *P. morganii*, *P. aeruginosa* and *P. putrida* were isolated from three species of European crayfish, as well as from North American red swamp crayfish (*Procambarus clarkii*) and spiny cheek crayfish (*Orconectes limosus*) (Vey *et al.*, 1974; Alderman and Polglase, 1988). *P. fluorescens* was isolated from crayfish showing clinical signs of infection (Alderman and Polglase, 1988). Infections are believed to be aquired by captive crayfish via surface wounds or infected food. Signs of infection include lethargy, trembling limbs, weakening and death. Pathogenicity is believed to be related to exotoxin or exoenzyme production by the bacteria (Alderman and Polglase, 1988). The effect of *Proteus* spp. and *Pseudomonas* spp. on wild populations is not known.

Bacteria of Penaeidae Boas, 1880 (genus *Penaeus*), Palaemonidae Rafinesque, 1815 (genus *Macrobrachium*) and Pandalidae Dana, 1852 (genus *Pandalus*)

Rickettsia-, chlamydia- and mycoplasma-like organisms

Rickettsia-like infections have been implicated in stained prawn disease (SPD) of spot

shrimp/prawn (*P. platyceros*) in British Columbia (Bower *et al.*, 1993, 1994a). Prawns fed infected tissue suffered 84% mortality within 2–4 months post-infection. Experimental transmission was also achieved via screened effluent from infected prawns and infectivity of the rickettsial-like organism was maintained, even after freezing at –15°C. Early stages appear as intense focal melanotic lesions in response to infection of the fixed macrophages between the tubules around the surface of the digestive gland and ventral cephalothorax. As the infection progresses, these lesions migrate towards the cuticle (Bower *et al.*, 1994a, 1996), where they become grossly visible as black 'pepper-like' stippling. Gonadal development and secondary sexual characteristics also appeared abnormal in infected prawns.

Chong and Loh (1984, in Lightner *et al.*, 1992b) reported rickettsial-like inclusions in the hepatopancreas of cultured banana shrimp/prawn (*P. merguiensis*). These appear similar to inclusions observed in red shrimp (*P. marginatus*) from Hawaii (Brock *et al.*, 1986b). Cytoplasmic microcolonies contain membrane-bound cells measuring 0.2–0.7×0.9–1.6 µm. Juvenile Pacific blue shrimp (*P. stylirostris*) fed infected red shrimp tissue suffered 85% mortality, but the pathogenicity of infection in wild shrimp populations is unknown (Brock *et al.*, 1986b).

A third rickettsial-like organism infects the midgut, hepatopancreas and caeca of giant tiger shrimp (*P. monodon*) in Malaysia (Anderson *et al.*, 1987) and Indonesia (Lightner *et al.*, 1992b). Microcolonies, 19–33 µm in diameter, are found inside large cytoplasmic vacuoles. The specific pathogenic effect of these microcolonies is undetermined due to concurrent infections with viruses, Gram-negative bacteria and epibionts (Anderson *et al.*, 1987; Brock and Lightner, 1990).

A rickettsial-like infection was also reported in white-leg shrimp (*P. vannamei*) suffering from Texas pond mortality syndrome (TPMS), also known as Texas necrotizing hepatopancreatitis (TNHP) (Krol *et al.*, 1991; Lightner *et al.*, 1992c). TPMS has caused up to 99% mortality in Texas shrimp ponds since the mid-1980s. The RLOs proliferate throughout the hepatopancreas, causing intense haemocyte infiltration, granulosis and necrosis. Clinical signs are lethargy, softened carapace and darkened gills. Two rickettsial-like organisms were described originally: one was rod-shaped (0.25×2.0–3.5 µm) and the other filamentous or helical (0.3×0.9 µm). Other spiral or filamentous microorganisms were found lying free (non-membrane bound) in the cytoplasm (Krol *et al.*, 1991). The rod and helical forms are now recognized as different developmental stages of the same organism (Lightner *et al.*, 1992c).

Gram-negative bacteria

Bacterial necrosis of a wide range of larval shrimp and prawns (*Penaeus* and *Macrobrachium* spp.) is attributed to *Vibrio* spp. and affects culture facilities worldwide (Lightner *et al.*, 1992b). The diseases, known as penaeid bacterial septicaemia, penaeid vibriosis, red-leg disease, 'sien dun' (Thai for black splint), luminescent vibriosis and seagull syndrome vibriosis ('sindroma gaviota'), often occur alongside *Pseudomonas*, *Aeromonas* and *Flavobacterium* spp. infections.

Luminescent *V. harveyi* and *V. splendidus* attach to the feeding appendages and oral cavity of larval giant tiger prawn and banana prawn, causing significant mortalities in Thailand and the Philippines (Baticados *et al.*, 1990; Lavilla-Pitogo *et al.*, 1990). Mortalities of giant river/freshwater prawn (*M. rosenbergii*) larvae in Thailand have also been attributed to *V. harveyi* (Tonguthai, 1992). The shrimp turn bluish, accumulate dense populations of epibionts and sink to the bottom of holding ponds. Susceptibility of penaeids is believed to vary between strains of *V. harveyi*, as well as *Penaeus* species; for example, Indian white prawn (*P. indicus*) postlarvae show no clinical signs or luminescence when challenged with up to 10^5 CFU/ml of *V. harveyi* for 96 h (Pillai and Jayabalan, 1993).

Mass mortalities of postlarval and juvenile Japanese shrimp (*P. japonicus*) occurring in Japan since 1981 are attributed to an unidentified *Vibrio* sp. (Takahashi *et al.*, 1985a; Egusa *et al.*, 1988; Lightner *et al.*,

1992b). Clinical signs include opacity of the sixth abdominal segment, brown spots on the gills and lymphoid organ (juveniles) and pale hepatopancreas (postlarvae). Aggregations of bacteria in the lymphoid organ become enveloped in melanized whorls surrounded by intense haemocyte infiltration. Formalin-killed *Vibrio* sp. have provided some protection under laboratory conditions (Itami *et al.*, 1989) and field trials with oxolinic acid or oxytetracycline medicated feed have been effective in controlling losses (Takahashi *et al.*, 1985b; Aoki, 1992) (see the section on chemotherapy under treatment and protection).

V. alginolyticus, *V. parahaemolyticus* and *Pseudomonas* sp. were isolated from market-size, moribund, giant tiger shrimp in Malaysia (Anderson *et al.*, 1988). Experimental investigations into the comparative virulence of the three *Vibrio* spp. showed *V. alginolyticus* to be most virulent to Japanese shrimp, while *V. parahaemolyticus* was most pathogenic to white shrimp (*P. setiferus*) (Leong and Fontaine, 1979, in Vera *et al.*, 1992).

A similar disease, known as seagull syndrome ('sindroma gaviota' or SGS-vibriosis), infects white-leg shrimp in Ecuador. The name reflects the aggregations of gulls and other seabirds which feed on dead and dying shrimp. Affected ponds are estuarine and peak mortalities coincide with periods of drought, when salinity and nutrient concentrations are high. Mortalities from SGS-vibriosis were reduced by a sucrose-based treatment of the pond water. It is believed that the sucrose provides a growth medium for less pathogenic *Vibrio* species, which outcompete non-sucrose-fermenting *V. parahaemolyticus* (Lightner *et al.*, 1992b).

Chitinolytic bacterial diseases, similar to those described from lobsters and crabs, have also been reported in shrimps and prawns (Lightner, 1988b). As in the other Crustacea, opportunistic infection by *Vibrio* spp. follows abrasion of the epicuticle and is especially pronounced in captive animals. Pathogenicity of shell lesions in wild-caught shrimps varies between host species, aetiologic agent and environmental conditions (Brock and Lightner, 1990).

Epibiont bacteria

Low levels of epibiont bacteria occur naturally on the surfaces of aquatic crustaceans and are usually controlled by preening. Since larval shrimp cannot preen, they are more susceptible to epibiont proliferation, which impedes feeding, respiration and swimming. Nutrient accumulation in holding facilities accelerates epibiont fouling, especially by species such as *L. mucor*, *Thiothrix* sp., *Flexibacter* spp., *Cytophaga* spp., *Flavobacterium* spp. (Lightner, 1988c) and a filamentous species tentatively identified as *Bacillus cereus* var. *mycoides* (Barkate *et al.*, 1974). In addition, certain non-filamentous bacteria may produce a mucoid layer which impedes gas exchange across the gills (Fisher, 1988b). The most serious fouling epibiont is *L. mucor*, a filamentous bacterium found on a wide range of macroscopic algae, as well as marine crustaceans (including lobsters, crabs and crayfish). Epicuticle colour changes from yellow-brown to brown-black as the bacteria proliferate and, although tissue penetration is rare, weakened hosts are more susceptible to invasive secondary pathogens. Conversely, infection by a primary pathogen is often indicated by reduced preening and epibiont proliferation (see section on viral infections of shrimps).

Bacteria of Brachyura Latreille, 1803

Rickettsia-, chlamydia- and mycoplasma-like organisms

To date, rickettsial-like organisms have been described from only two species of crabs, but asymptomatic infections most likely have a broad host distribution. A *Rickettsia*-like organism isolated from Mediterranean shore crab (*C. mediterraneus*) from the Mediterranean coast of France caused pathogenic lesions in the hepatopancreas, gill, gonad and intestinal connective tissue of experimentally inoculated crabs (Pappalardo and Bonami, 1980). Feulgen-positive microcolonies (10–20 µm in diameter) containing rod-shaped organisms

(0.7×2 μm) with a plasma membrane and cell wall were found in cytoplasmic vacuoles in these tissues. No evidence of clinical infections in wild populations of this shore crab have been reported (Brock and Lightner, 1990)

A rickettsial-like infection was also reported from a single blue king crab (*P. platypus*) in Alaska which had massive infiltration of the hepatopancreatic tubules, inhibited ovarian development and a disrupted moult cycle (Johnson, 1984). The microcolonies (10–40 μm) contained oval, weakly Feulgen-positive microorganisms (0.3×0.6–1.0 μm) and infected the hepatopancreatic epithelia. Despite this obvious pathology, the effect on wild populations is unknown.

In contrast, a *Chlamydia*-like organism in Dungeness crab (*C. magister*) from Washington State was related to significant mortalities. Winter prevalences ranged from 6 to 13% between 1978 and 1982 and all infections were heavy, suggesting a correlation between low water temperature and pathogenicity of the infection. Intense haemocyte infiltration was associated with infected fixed phagocytes and other hypertrophied and necrotic ecto- and mesodermal tissues (Sparks *et al.*, 1985). Stages resembling elementary bodies were found in cells with marginally displaced nuclei and few cytoplasmic organelles. Reticulate, intermediate and ellipsoidal stages were found in less severely disrupted cells.

Gram-positive bacteria

A bacterium identified as *Streptococcus faecalis* var. *liquefaciens* was reported in Mediterrancean shore crabs from the French Mediterranean coast (Pappalardo and Boemare, 1982). Infected crabs were weak and had systemic infections by Gram-positive cocci and diplococci surrounded by intense haemocyte infiltration. The bacteria were identified as a pathogenic variant of *S. faecalis*, a faecal contaminant found at a nearby sewage system. No similar infections were found in crabs collected further from the sewage outfall area.

Gram-negative bacteria

Chitinolytic bacteria, such as those described for shrimps and lobsters, also affect crabs, especially those held in captivity (Brock and Lightner, 1990; Messick and Sindermann, 1992). *Vibrio* spp. and *Pseudomonas* spp. have been isolated from Caribbean spiny lobster (*P. argus*) (Sindermann and Rosenfield, 1967), spotted spiny lobster (*P. guttatus*) (Iversen and Beardsley, 1976), red king crab (*P. camtschatica*), blue king crab (*P. platypus*) (Bright *et al.*, 1960, in Sindermann and Rosenfield, 1967), blue crab (*C. sapidus*) (see summary of Messick and Sindermann, 1992), Mediterranean shore crab (Sindermann, 1977; Baross *et al.*, 1978), Atlantic rock crab (*C. irroratus*) (Young and Pearce, 1975), Tanner crab (*C. tanneri*) (Baross *et al.*, 1978), snow crab (*C. opilio*), Florida stone crab (*Menippe mercenaria*), black-fingered/black-clawed/Atlantic mud crab (*P. herbstii*), batwing coral crab (*Carpilius corallinus*) and blue land crab (*Cardisoma guanhumi*) (Iversen and Beardsley, 1976) and red deep-sea crab (*G. quinquedens*) (Young, 1989). Baross *et al.* (1978) characterized 60 chitinolytic isolates from crab shell lesions. Tanner crabs were affected predominantly by luminescent *Photobacterium* spp., *Moraxella* spp. and *V. anguillarum*. All chitinolytic isolates from Dungeness crabs resembled *V. anguillarum*. Young (1989) linked the severity of infections observed in wild red deep-sea crabs to the duration of the intermoult period, where large crabs accumulated sufficient adhesions to impede moulting and die. This may also be the case for other crustaceans (Sindermann, 1989).

Epibiont bacteria

Gram-negative bacteria, predominantly *L. mucor*, have been found on wild and captive crabs (Johnson *et al.*, 1971) including long-armed hermit crabs (*Pagurus longicarpus*), green crabs (*C. maenas*), Atlantic rock crab, Dungeness crab, northern stone crab (*L. maia*) and daggerblade grass shrimp (*Palaemonetes pugio*) (Johnson *et al.*, 1971; Fisher, 1977, 1988b). Although generally

not a problem for healthy crustaceans, Fisher and Wickham (1976) found a direct correlation between fouling of eggs and larvae of Dungeness crabs collected from San Francisco Bay and survival rates under holding conditions. The bacteria form filamentous outgrowths from the cuticular surfaces of the carapace, gills and egg masses. The significance of these epibionts to population fluctuations of Dungeness crabs, however, is unknown (Brock and Lightner, 1990).

Bacteria of Echinoidea Mortensen, 1907

Gram-negative bacteria

One of the few bacteria-related diseases reported from echinoderms is 'bald sea urchin disease' which affects at least nine species of echinoid, including red sea urchin (*Strongylocentrotus franciscanus*), purple sea urchin (*S. purpuratus*) and fragile red urchin (*Allocentrotus fragilis*) from California, and black urchin (*Arbacia lixula*), pencil-spine urchin (*Cidaris cidaris*), northeastern Atlantic purple sea urchin (*Paracentrotus lividus*), edible sea urchin (*Echinus esculentis*), flat green sea urchin (*Psammechinus miliaris*) and the violet sea urchin (*Sphaerechinus granularis*) from the western Mediterranean and Atlantic coast of France (see review by Jangoux, 1990). Gilles and Pearce (1986) examined 14 strains of bacteria from diseased purple sea urchins (*S. purpuratus*) and concluded that bald sea urchin lesions were caused by Gram-negative *V. anguillarum* and *A. salmonicida*. The lesions (also known as 'red spot disease' in California) start as green-purple discoloration of the epidermis at the base of some spines. The underlying epidermis becomes necrotic and sloughs off, leaving areas of exposed test. This is repairable if less than 30% of the total body is affected (Maes and Jangoux, 1984); however, extensive lesions, or perforation of the test, leads to mortality. Lesions in *S. droebachiensis* are believed to be due to chronic secondary infections (Roberts-Regan *et al.*, 1988), rather than pathogen-specific bacteria (Jones and Scheibling, 1985).

Gram-positive bacteria

Black sea urchin plague, a serious disease of black sea urchins (*Diadema antillarum*) in the Caribbean (Lessios *et al.*, 1984), was linked to isolates of *C. perfringens* and *C. sordelli*, Gram-positive, anaerobic, rod-shaped bacteria. The first clinical sign of the plague is the appearance of a colourless mucus over the surface of the spines. The urchins stop responding to tactile stimuli and may lose spines. Bare patches of test developed penetrating lesions, especially along the interambulacrae, and mortalities occurred approximately 10 days after the appearance of clinical signs (Hughes *et al.*, 1985). The Caribbean black sea urchin population declined by 93–99% over a period of 2 years. No other sympatric sea urchin species were affected (Liddell and Ohlhorst, 1986).

Fungal Infections

Fungi of Gastropoda Cuvier, 1797

Various fungal diseases exist that affect cultured and wild abalone, including foot tubercles and shell mycosis. Siebold's abalone (*H. sieboldii*) held in a live-storage facility in Japan were found to have tubercle-like swellings (up to 5 mm in diameter) in the mantle and foot. Examination revealed infection by *Haliphthoros milfordensis*, a ubiquitous estuarine and marine fungus (Hatai, 1982).

In Jacks Bay on the South Island of New Zealand, a mild fungal infection was also noted in an underweight paua shell (*H. iris*) (about 47 mm shell length) from an abalone culture facility. The fungal agent was not identified, but was allied presumptively with the Class Deuteromycotina. Similar infections were found in the queen paua shell (*H.* [*Sulculus*] *australis*) and the virgin paua shell (*H. virginea*). Conchiolin and nacreous deposits were observed on the inner shell surface near the shell apex, with some shell lesions extending to the foot-muscle attachment site. In severe cases, the lesions penetrated through to the periostracal (external) surface. Although hyphae

were found throughout the shell matrix, none were observed in the soft tissues and abalone condition appeared unaffected (Friedman *et al.*, 1997b; Grindley *et al.*, 1998). Diggles and Oliver (2005) have identified these infections as a potential problem for abalone culture due to the progressive nature of shell destruction as the abalone grows.

Fungi of Bivalvia Linné, 1758

The phycomycete fungus, *Ostracoblabe implexa*, is responsible for severe shell damage in European oysters (*O. edulis*), known as 'maladie de la charnière' (hinge disease), 'maladie du pied' (foot disease, erroneously), shell disease and Dutch shell disease (Fig. 20.15a,b). Less severe infections occur in the Pacific oyster (*C. gigas*), hooded rock oyster (*Saccostrea cucullata*) in India and Portugese oyster (*C. angulata*) and the edible cockle (*Cardium edule*) (Korringa, 1951; Cole and Waugh, 1956; Quayle, 1969; Li *et al.*, 1983; Alderman, 1986; Raghukumar and Lande, 1988) in Europe. Infections first appear as raised white spots with translucent centres which coalesce into conchiolin warts on the inner shell surface. In European oysters, surface irritation results in heavier conchiolin deposition than in other infected bivalves (Alderman, 1982). The protein rich conchiolin accelerates proliferation of the fungus and thickening of the shell margin and hinge ('maladie de charnière') or adductor muscle attachment surface ('maladie du pied'). Fungal growth is restricted to warmwater temperatures (>20°C), so severe infections are more common in shallow water. Proliferation of *O. implexa* over a period of several weeks can lead to shell destruction and the death of the oyster. Microscopic examination of decalcified shell reveals a dense mycelial network of hyphae 1.5–2.5 µm in diameter. Small oval dilations (chlamydospores) occur at irregular intervals (40–100 µm) along the hyphae and septa only appear in dying mycelia (Alderman, 1982) (Fig. 20.16a,b). Mantle tissues in direct contact with affected shell may become metaplasic, but are not infected by the fungus. In Atlantic Canada, bivalve species growing beside infected European oysters show no evidence of infection (Li *et al.*, 1983).

The oomycete fungus, *Sirolpidium zoophthorum*, is well documented as the

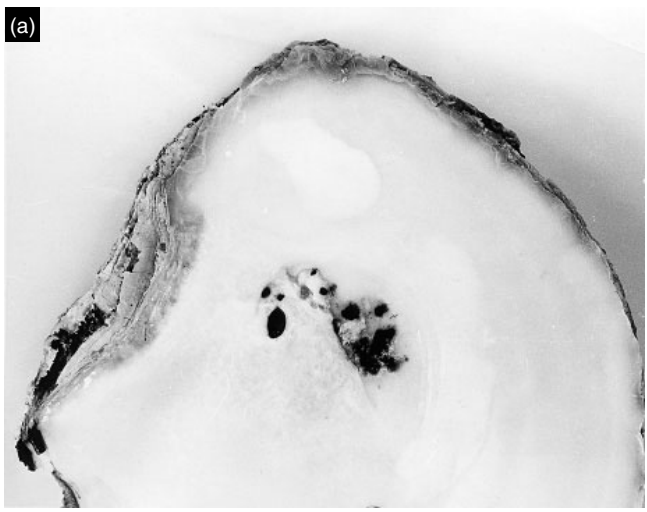


Fig. 20.15. (a) Conchiolin warts in the shell of *Ostrea edulis* infetected by the fungus *Ostracoblabe implexa* ('shell disease'). Note lesions at the adductor muscle attachment point of the shell. Photograph courtesy of M.F. Li.

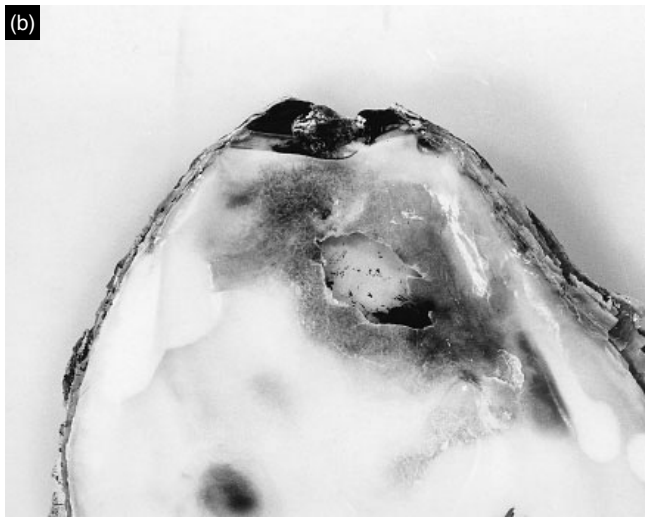


Fig. 20.15. (b) Advanced destruction of the nacre layer of the shell of *Ostrea edulis* due to infection by the fungus *Ostracoblabe implexa*. Photograph courtesy of M.F. Li.

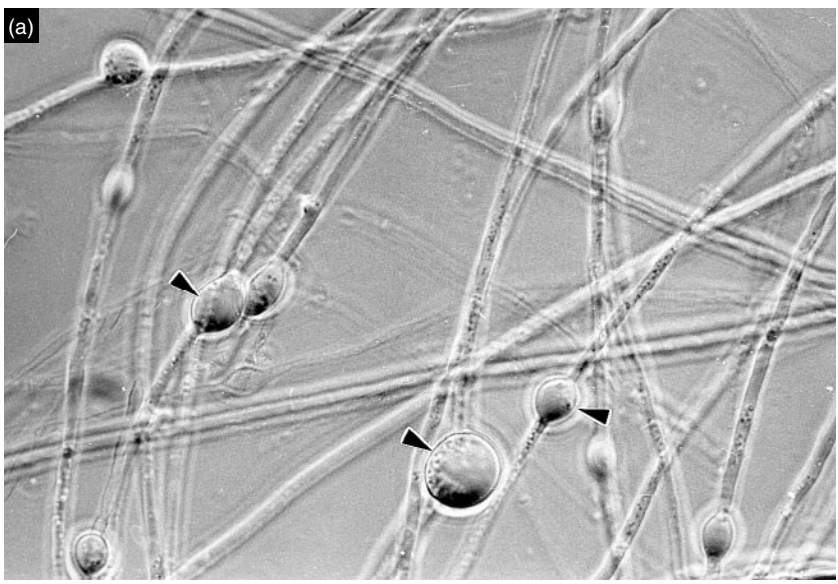


Fig. 20.16. (a) Chlamydospores (4–6 µm in diameter) (arrows) of *Ostracoblabe implexa* forming on aseptate hyphae in sterile seawater culture. Photograph courtesy of M.F. Li.

cause of larval mycosis in hatchery-reared American oysters (*C. virginica*) and hard-shell clams (*M. mercenaria*) (Davis *et al.*, 1954; Vishniac, 1955; Johnson and Sparrow, 1961). Veliger and postmetamorphic larvae up to 0.4 mm in length are particularly susceptible and can suffer over 90% mortality within 48 h. *S. zoophthorum* is cultured easily on nutrient agar, producing a branched, septate thallus (10–15 µm in diameter) which thickens to 82 µm in diameter at maturity. Swollen terminal cells produce



Fig. 20.16. (b) Septate hyphae of *Ostracoblabe implexa* forming on dying mycelia. Photograph courtesy of M.F. Li.

sporangia. These produce discharge tubes (up to 142 µm in length and 5 µm in diameter) through which biflagellate zoospores (2×5 µm) are released (Vishniac, 1955).

Another fungus affecting oyster hatchery production was described as 'digestive tract impaction'. Pacific oyster larvae which failed to complete metamorphosis within 12 days of hatching showed over 90% mortality (Leibovitz *et al.*, 1978; Elston, 1980a). The infection was characterized by erosion of the mantle and velar epithelia and occlusion of the stomach by thick-walled spheres. The intestine was dilated or ruptured. The spheres were identified tentatively as a *Dermocystidium*-like fungus, a phycomycete fungus or a *Hyalochlorella* colourless alga. No further outbreaks have been reported since the original description from four hatcheries in Washington State in the late 1970s.

A disease of wild and cultured quahogs (hard-shell clam) in the Gulf of St Lawrence, Canada, was described originally as an *Olpidium*-like, Chytrid (Phycomycete) fungus (Drinnan and Henderson, 1963). Whyte *et al.* (1994) found thraustochytrid-like ultrastructural characteristics; thus, the fungal-like agent was designated 'quahaug parasite unknown' (QPX). The organism of uncertain taxonomic position was identified tentatively as an unusual member of the phylum Labyrinthulomycota (= Labrynthomorpha), possibly in the family Thraustochytriidae (Maas *et al.*, 1999). This was confirmed by the phylogenetic molecular analysis of Ragan *et al.* (2000). Analysis by Maas *et al.* (1999) of the ultrastructure reinforced the molecular attribution and suggested that QPX was a primitive member of the phylum because of the absence of typical sagenogenetosomes (sagenogens) and ectoplasmic nets in the clam host. However, Kleinschuster *et al.* (1998) reported the development of an ectoplasmic net in cultured QPX when transferred to sterile seawater. Thus, ectoplasmic net characteristics of cultured QPX remain open to debate.

Initially reported from wild and hatchery-reared hard-shell clams in the Gulf

of St Lawrence, Canada (Drinnan and Henderson, 1963; Whyte *et al.*, 1994), it has been reported subsequently in the same species in New Brunswick and Nova Scotia, Canada, and in Massachusetts, New Jersey, New York and Virginia, USA. In Virginia, QPX was not found in hard-shell clams from Chesapeake Bay but was present in cultured clams from three coastal embayments (Ragone Calvo *et al.*, 1998). A morphologically similar parasite has also been reported from European carpet clams (*R. decussates*) from Portugal (Azevedo and Corral, 1997).

Ford *et al.* (2002) and Ragone Calvo *et al.* (2007) indicate that different strains of hard-shell clams vary in their susceptibility to infection, disease and mortalities caused by QPX. *M. mercenaria* var. *notata* (a phenotypic strain of hard-shell clam developed for culture purposes) appeared to be most susceptible. QPX is most prevalent in cultured clams being held in the hatchery or nursery or, occasionally, in densely set natural populations. It was suggested to be the primary cause of significant wild stock mortalities in New Brunswick in 1959 (Drinnan and Henderson, 1963), but no mortalities attributed to QPX have occurred in wild stocks in Atlantic Canada since (Bacon *et al.*, 1999; MacCallum and McGladdery, 2000). QPX was associated with 80–90% mortalities in juvenile clams (up to 30 mm shell length) in a nursery and up to 100% in hatchery broodstock on Prince Edward Island (Whyte *et al.*, 1994; Bacon *et al.*, 1999). It has also caused severe mortality (80–95%) in cultured clams in Massachusetts (Fraser, 1996; Smolowitz and Leavitt, 1997; Smolowitz *et al.*, 1998), New Jersey (Ford *et al.*, 2002) and eastern Virginia (Ragone Calvo and Burreson, 2002), where in 2002 it threatened the New York hard-shell clam fishery.

QPX disrupts the connective tissue throughout the body of the clam. Developmental stages range from dense spherical bodies (5–12 µm in diameter) resembling cysts, to large endosporulating stages (>100 µm in diameter) containing daughter cells at various stages of development (Fig. 20.17a–c).

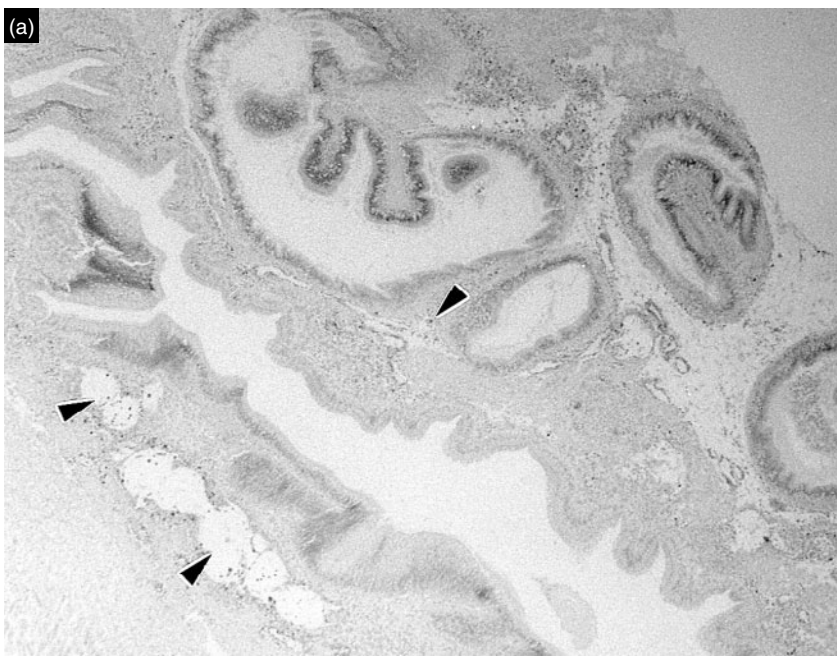


Fig. 20.17. (a) Massive infiltration and tissue lysis (arrows) of *Mercenaria mercenaria* infected by QPX ('quahaug parasite X'), a chytrid-like parasite (H & E, ×25).

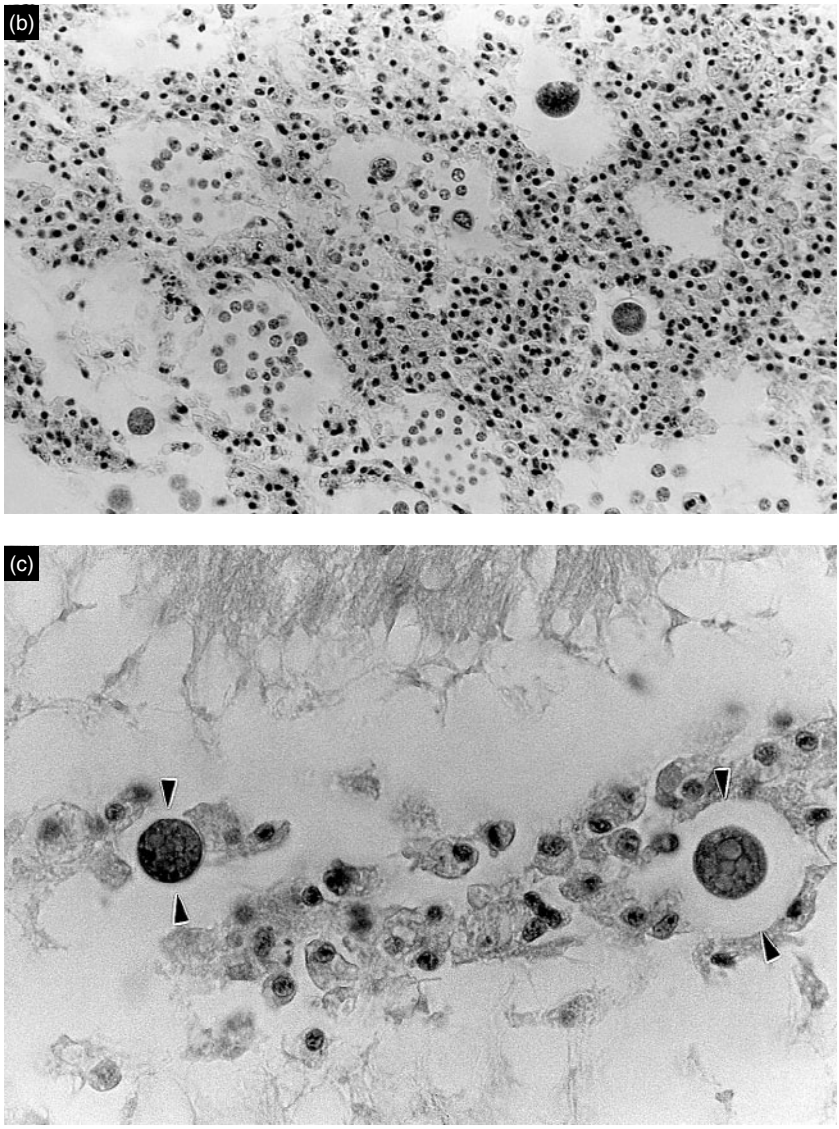


Fig. 20.17. (b) Endosporulating vegetative stages and thick-walled spore-like stages of QPX surrounded by inflammatory haemocytes in *Mercenaria mercenaria* connective tissues (H & E, ×250). (c) Spores of QPX showing development of thick laminate walls (indicated by encapsulating haemocytes) (arrows) (H & E, ×630).

Cell walls range from membranous to thick, translucent cysts and most stages are associated with varying degrees of tissue lysis (Whyte *et al.*, 1994), which appear to be linked to ectoplasmic-like extrusions from the cell wall. Various stages of development are found extracellularly, along with debris and haemocytes from lysed lesions. Other bivalve species (American oysters [*C. virginica*], bay scallops [*A. irradians*] and blue

mussels [*M. edulis*]) held in close proximity to infected clams showed no evidence of QPX transmission. Aggregations of vegetative stages of the pathogen are surrounded by clear histolytic zones. Peripheral necrotic haemocytes indicate the possible production of a toxic substance with lytic activity. Mortality is most severe in the spring and summer months in clams at least 1 year old. In the eastern USA, this is associated with gross lesions (swellings and nodules) in the mantle of infected clams. Such lesions have not been observed in infected Canadian populations. Smolowitz *et al.* (1998) reported that QPX-infected clams grew more slowly and had a lower condition index than uninfected clams. Laboratory studies indicate that QPX has a direct life cycle and is transmitted directly between clams within 3 months of exposure (Smolowitz *et al.*, 2001). The mucoid material produced by QPX appears to resist defensive reactions and may prevent phagocytosis by clam haemocytes, thus representing an important virulence factor (Anderson *et al.*, 2003). While many aspects of the basic biology and epizootiology of QPX disease remain unknown, observations to date suggest that genetic variability in the host and/or in the QPX pathogen is responsible for differences in susceptibility to infection (Ragone Calvo and Burreson, 2002; Ragone Calvo *et al.*, 2003).

Fungi of Cephalopoda Cuvier, 1797

Polglase *et al.* (1984) described the first fungus from a cephalopod. *Cladosporium sphaerospermum* was isolated from experimentally induced wounds in the curled octopus (*Eledone cirrhosa*). Mycelia penetrated the underlying dermis and radiated out from the surface of the wound; however, the possibility that *C. sphaerospermum* was a secondary saprobiont rather than a primary pathogen could not be ruled out (Polglase *et al.*, 1984).

Two thraustochytrid-like organisms, *Schizochytrium* sp. and *Ulkenia ammoeboidea*, were associated with an ulcerative condition in wild-caught curled octopus from Scotland (Polglase, 1980; Alderman, 1982). Early clinical signs were grey patches of inactivated chromatophores, followed by ulcerated white lesions which spread and led to death within 2–3 days. All infected octopuses died within approximately a week in captivity. One of the two organisms isolated from the lesions was characterized by rounded cells (6 µm in diameter) which formed tetrads, diads or combinations of both. The second organism showed similarities to the genus *Labyrinthula* sp. (Polglase, 1980).

Jones and O'Dor (1983) described gill lesions in captive short-finned squid (*Illex illecebrosus*) from Nova Scotia, which they attributed to another thraustochytrid-like organism. All infected squids died. Tissue sections contained spherical cells (up to 7 µm in diameter), with irregular bipartitioning of the vegetative thallus into diads, triads or tetrads (Jones, 1981, cited in Alderman, 1982). Large aggregations caused cell lysis of the gill epidermis and were associated with an ectoplasmic net. This is similar to tissue damage described for other thraustochytrid infections (Perkins, 1973).

Fungi of Nephropidae Dana, 1852 (genus *Homarus* Weber, 1795)

Three fungal diseases have been reported from captive lobsters. *Lagenidium* sp. (Oomycete) infects cultured American lobster (*H. americanus*) and European lobster (*H. gammarus (= vulgaris)*) larvae in California (Nilson *et al.*, 1976) and appears related to *Lagenidium callinectes* in crabs and shrimps (Fisher, 1988c). The fungus penetrates the carapace of young lobsters and spreads through the soft tissues, giving them an opaque, milky appearance. Light microscopy reveals dense networks of finely branched mycelia with globule-filled hyphae. Sporangia are formed at the swollen tips of terminal hyphae. These produce long discharge tubes which extend out of the host's body and terminate in a characteristic vesicle. The presence of this vesicle differentiates *Lagenidium*

from other marine oomycetes, *S. zoophthorum* and *Haliphthoros* spp. Although the measurements of the species infecting lobsters are not given (Fisher, 1988c), it is described as similar to *L. callinectes*, which is known to form discharge tubes 80–1500 μm in length with terminal vesicles up to 2000 μm in diameter (Hatai, 1989). Biflagellate zoospores are partially produced within the sporangium but complete maturation within the terminal vesicle. Proliferation of infection was related to excessive stocking density, high water temperature and low water exchange (Fisher, 1988c).

Another oomycete fungus, *H. milfordensis*, infects a wide range of crustacean hosts including juvenile (postlarval) American and European lobsters (Fisher *et al.*, 1975; Fisher, 1988d; Brock and Lightner, 1990). The fungus causes extensive damage of the exoskeleton, melanization and adhesion of the underlying integuments. Infections in captive lobsters inhibit moulting and result in high mortality. The significance of *H. milfordensis* to wild lobsters, however, is unknown. The fungus has branched, aseptate hyphae (10–25 μm in diameter) with occasional bulbous swellings (40 μm in diameter). Sporangia develop from elongate or spherical gemmae and produce discharge tubes up to 725 μm in length. Biflagellate zoospores (6×8 μm) develop and mature within the hyphae prior to release via the discharge tube (Tharp and Bland, 1977).

Deuteromycete *Fusarium* spp. are responsible for 'burn spot' or 'black-gill' disease in a wide range of crustaceans including juvenile lobsters (Lightner and Fontaine, 1975; Fisher *et al.*, 1978; Alderman, 1981). Infections with *F. solani* caused mass mortalities of juveniles at a New York lobster hatchery where hyphal and conidial stages were reported from thoughout the gill lamellae and epicuticle. Early spot formations progressed from white to orange to brown-black as the infection developed. Micro- (single-celled) and macroconidia (two- to ten-celled) were observed at the tips of infected gill lamellae and were characteristically elongate and curved. Mortality occurs before or during moulting (Lightner and Fontaine, 1975) but, as with other oomycetes, the effect of infection on wild populations remains unknown.

'Leopard lobster' (mottling disease) of American lobsters in Maine, USA, originally thought to be aberrant coloration, has since been linked to a thraustochytrid-like fungus infection (Unestam, 1973; Alderman, 1976, 1982). Unlike other fungal infections, mottling disease has only been observed in wild lobsters and does not appear to be related to culture or holding conditions. Yellow-beige patches on the cuticle correspond to necrotic underlying tissues and advanced infections show blistering at the cuticle surface. The necrotic tissues contain PAS-positive, thick-walled spheres, 30–60 μm in diameter. Since normal lobster carapaces may also show yellow patches (as well as red and blue), infection must be confirmed by observation of the pathogen.

Fungi of Nephropidae Dana, 1852 (genera *Astacus, Pacifascticus, Camabrus, Cherax, Austropotambius* and *Orconectes*)

One of the best known fungal diseases of crustaceans is crayfish plague ('krebspest', 'kraftpest', 'la peste' or crayfish aphanomyciasis), caused by *Aphanomyces astaci* (Saprolegniales). This fungus is common in North American crayfish (signal crayfish [*Pacifastacus leniusculus*] and red swamp crayfish/crawfish [*Procambarus clarkii*]), where it causes superficial lesions, but is not associated with epizootic mortality. In contrast, crayfish in Europe (noble/broad-fingered crayfish [*Astacus astacus*], Danube/Galician/Turkish or narrow-clawed crayfish [*A. leptodactylus*] and freshwater white-clawed crayfish [*A. pallipes*]) have been decimated by *A. astaci* infections (Unestam, 1973; Alderman and Polglase, 1988). The fungus was introduced into Italy in the 1860s with North American crayfish and spread from there throughout Europe, Russia, Scandinavia and, recently, to the UK (Alderman and Polglase, 1988). Ten species of crayfish from Japan, Australia and New Guinea also show susceptibility (Unestam, 1975).

Fungal hyphae penetrate non-calcified sections of the cuticle and spread systemically, often along the nerve cord. Focal melanization in North American crayfish limits the spread of mycelia through the endocuticle. European crayfish, however, show negligible melanization and die within 2 weeks of exposure. The fungus is characterized by delicate, branching, aseptate hyphae (7–9 μm in diameter) with granular cytoplasm (Alderman and Polglase, 1986, 1988). Zoosporangia are myceloid.

Another fungal infection of European crayfish, principally the noble crayfish and spiny cheek crayfish (*O. limosus*), is 'burn spot disease' caused by *Ramularia astaci*, *Didymaria cambari* (Hyphomycete) or *Fusarium* spp. (Phycomycete). Prevalences of 2–85% are widespread in Europe and cause significant mortalities (Järvekulg, 1958, cited in Sparks, 1985). *R. astaci* causes lesions over 10 mm in diameter which expose or weaken the exoskeleton. Hyphae invade the gills and muscular tissue, forming conidia around the edges of lesions (in both dead animals and moulted exoskeletons). *D. cambari* causes similar, but smaller, lesions (5 mm) and infects the uropods (Alderman and Polglase, 1988).

Fungi belonging to the Oomycete group, *Saprolegnia* spp., affect red swamp crayfish aquaculture in the USA (Avault and Huner, 1985). Infections of ova are especially serious in overcrowded conditions which inhibit normal preening behaviour of the female crayfish and removal of the dead or dying eggs.

Fungi of Penaeidae Boas, 1880, and Pandalidae Dana, 1852

'Black gill disease' is caused by Deuteromycete fungi belonging to the genus *Fusarium* (principally *F. solani*). The disease affects Japanese shrimp (*P. japonicus*), pink shrimp (*P. duorarum*), white shrimp (*P. setiferus*), brown shrimp (*P. aztecus*), white-leg shrimp (*P. vannamei*), blue-leg shrimp (*P. stylirostris*) and Californian brown shrimp (*P. californiensis*) (Brock and Lightner, 1990) and can cause significant mortality. Under intense culture, over 90% cumulative mortalities of Japanese and Californian brown shrimp may occur over 4–5 months (Lightner *et al.*, 1979). Infections are initiated via epicuticle abrasion and first appear as raised lesions on the gills, eyes, appendages or other integument surfaces (Alderman, 1981). Gill squashes show masses of septate hyphae with boat-shaped macroconidia (Lightner and Fontaine, 1975). Histologically, early stages of infection elicit intense haemocyte infiltration and melanization of the invasive hyphae. As the disease progresses, haemocyte concentrations decrease, the mycelia spread and secondary infections may occur (Solangi and Lightner, 1976; Lightner, 1988d). Treatment and control of *F. solani* are difficult since it is a ubiquitous soil or waterborne saprobiont.

Nauplii, zoea and mysis stages of penaeid shrimps are also infected by *L. callinectes* (Couch, 1942), *L. scyllae* (Bian *et al.*, 1979), *S. zoophthorum* (Vishniac, 1955) and *Haliphthoros* spp. (Hatai *et al.*, 1980). According to Brock and Lightner (1990), *L. scyllae* and *L. callinectes* are not clearly distinct and require more comparative investigation. *L. callinectes* is distinguished from *Sirolpidium* by the absence of terminal sporangia on *L. callinectes* discharge tubes. *Sirolpidium* also has a highly variable hyphal width (7–40 μm) compared with that of *L. callinectes* (5–14 μm) (Vishniac, 1955). Several culture characteristics can also be used to distinguish *Lagenidium*, *Atkinsiella*, *Sirolpidium* and *Haliphthorus* (Hatai, 1989). *Lagenidium* forms vesicles within which zoospores form, whereas the other three genera use parts of the thallus for zoospore development. *Lagenidium* zoospores are larger (8–10 μm) than those of the other genera (5–7 μm). Differentiation between *Sirolpidium* spp., *H. milfordensis* and *Haliphthoros* spp. (as well as *Atkinsiella* from crabs, described below) requires experience (Hatai, 1989; Brock and Lightner, 1990).

Nauplii and protozoeal stages of penaeids are infected predominantly by *L. callinectes*, while later stages appear more susceptible to *Sirolpidium* sp. (Lightner,

1983). Infected larvae show hyphal proliferation throughout the body, resulting in 100% mortality (Lightner, 1981); however, the significance of these fungi to wild shrimp populations is unknown.

Fungi of Brachyura Latreille, 1803

Chitinolytic fungal disease ('black mat syndrome') due to *Trichomaris invadens* was described originally as the Coelomycete, *Phoma fimeti* (Van Hyning and Scarborough, 1973) and is the only Ascomycete infection reported from crustaceans. The bairdi and tanner crab (*C. bairdi*) and, to a lesser extent, the snow crab (*C. opilio*) and grooved tanner crab (*C. tanneri*) (Sparks and Hibbits, 1979) are infected off Alaska (Sparks, 1985), British Columbia (Bower *et al.*, 1994a) and the east coast of Newfoundland (Dr J. Brattey, Northwest Fisheries Research Centre, Department of Fisheries and Oceans, St John's, Newfoundland, 1995, personal communication). Early signs are small black spots (fungal hyphae and thick-walled fruiting bodies) on the dorsal carapace (or limbs and eyestalks). These spread and coalesce until the entire surface is covered (hence the name 'black mat syndrome/disease') and may penetrate the carapace, invading the soft tissues. In the bairdi crab, soft-tissue infection is severe, spreading through the musculature and internal organs with a negligible defence response. Gut, midgut, hepatopancreas, antennal gland, gonad and mandibular organs generally are not infected. Severe infections impede moulting and are fatal, which suggests a potentially significant impact on wild populations (Sparks, 1982). Certain offshore populations surveyed in 1980 and 1981 had prevalences of 65% (Hicks, 1982) and 94.7% of barren females surveyed in 1981 were infected; however, a correlation between fecundity and *T. invadens* could not be determined conclusively. The intramatricial hyphae are non-pigmented and approximately half the diameter of surface hyphae (5.0–5.5 μm). *T. invadens* has not been cultured successfully on standard mycological media.

L. callinectes, originally described by Couch (1942) from the eggs of blue crab (*C. sapidus*), has since been reported from many crustacean species. The fungus has been isolated from cultured larvae, the surface of algae and up to 25% of wild crab egg masses, suggesting a significant effect on fecundity (Brock and Lightner, 1990). In Chesapeake Bay, Rogers-Talbert (1948) found up to 95% of crab egg masses to be infected. Bland and Amerson (1974) found up to 50% of the eggs in an infected egg mass to be non-viable. No infections have been reported on the west coast (Fisher and Wickham, 1976), although Dungeness crabs are known to be susceptible under hatchery conditions (Armstrong *et al.*, 1976). *L. callinectes* has branched mycelia with globule-filled intramatricial hyphae (5–14 μm in diameter). Vegetative fruiting structures develop into zoosporangia on extramatricial hyphae and release biflagellate zoospores (3–9×5–12 μm). These encyst on the surface of crab eggs or larvae, and the hyphae penetrate the outer coat, destroying the egg or larva within 48 h (Bland and Amerson, 1973).

The oomycetes *Atkinsiella dubia* and *A. hamanaensis* (Haliphthoraceae) are reported from a range of cultured and experimentally infected crabs (Atkins, 1954; Sparrow, 1973; Bian and Egusa, 1980), as well as brown shrimp (*P. aztecus*) (Lightner, 1981). *A. hamanaensis* causes mortalities in Mangrove crabs (*Scylla serrata*) cultured in Japan and is particularly pathogenic to eggs and larvae (Bian *et al.*, 1979; Bian and Egusa, 1980). The effect on wild populations is unknown. *A. hamanaensis* is characterized by broad (12–40 μm in diameter), sparsely septate hyphae which terminate in short or elongate zoosporangia (10–30×50–100 μm). Zoospores measure 6.3×4.5 μm (Bian and Egusa, 1980). *A. dubia* forms branched hyphae ranging from 27 to 50 μm in diameter, with bulbous sporangia up to 100 μm in width.

Fungi of Echinoidea Mortensen, 1907

Jangoux (1990) cites several references to a 'very peculiar disease' of Antarctic sea

urchins (genera *Rhynchocidaris* and *Ctenocidaris*). The aetiologic agent, *Echinophyces mirabilis*, is believed to be a fungus (Mortensen, 1909, cited in Jangoux, 1990) and affects the primary spines by changing their shape and increasing fragility. The infection also causes displacement of the genital pore and apical plate, but does not castrate the sea urchin. Other spine infections by fungal-like agents have been reported (Johnson and Chapman, 1970) but the causative agents are unknown.

Pathogenesis and Immunity

Bivalvia Linné, 1758

Immunity and resistance

Knowledge about the immunological mechanisms in bivalves and other shellfish has increased dramatically over the past 15–20 years, especially in the area of biomolecular factors. For a detailed summary of this work, the review of Bachère *et al.* (1995) is highly recommended, for both molluscan and crustacean hosts. An overview of this progress of knowledge, to date, is provided here.

The susceptibility of larval and post-metamorphic bivalves to infection by opportunistic microbial organisms is well documented, as is the relative non-pathogenic effect of many similar or identical organisms in larger juveniles and adults of the same species (Davis *et al.*, 1954; Tubiash *et al.*, 1965, 1970; Tubiash, 1975; Elston, 1980a; Brown, 1981; Elston *et al.*, 1981; Jeffries, 1982; Elston and Lockwood, 1983; Nottage and Birkbeck, 1986; Bower, 1987a,b; Lodeiros *et al.*, 1987; Dungan and Elston, 1988; Leibovitz, 1989; Nottage *et al.*, 1989; Nicolas *et al.*, 1992a; Le Deuff *et al.*, 1994; Renault *et al.*, 1994). Most opportunistic infections of bivalve larvae result in mass mortality; thus, acquired immunity, which presupposes survival of the initial challenge, and development of biomolecular defence recognition factors (Feng, 1988) are unlikely. Inherent immunity in bivalves is rapid for certain diseases, e.g. Malpeque disease of American oysters, where survivors of initial mortalities are capable of conferring immunity directly to the next generation (Mr Roy E. Drinnan, Shellfish Aquaculture Consultant, Musquodoboit Harbour, Nova Scotia, 1995, personal communication) and *Roseovarius* oyster disease (= JOD) (Farley *et al.*, 1996; Lewis *et al.*, 1996). Other oyster diseases, such as MSX, caused by the protistan parasite *Haplosporidium nelsoni*, take longer to develop consistent heritable defence mechanisms (Ford, 1988), possibly due to pathogen-specific resistance in the face of multiple pathogen challenges (Ford and Haskin, 1988, 1995). The factors rendering larvae more susceptible to opportunistic microbial infections than adults appear to be predominantly husbandry related. Mass mortalities can be reduced or eradicated by improving the quality of food, optimizing feed conversion and holding densities, and monitoring for signs of mortality event onset. Larval mortalites in the wild are rarely observed or studied; however, sporadic failures in recruitment indicate the possibility that open-water conditions that predispose larvae to opportunistic infections may also occur. Due to the complexity of environmental influences on susceptibility of larval bivalves (as well as shrimp) to opportunistic infections, the question of whether or not larval humoral defence mechanisms are as developed as in adults has not been clearly determined. The genetic factors regulating development of specific disease resistance in bivalves are now an area of intense investigation (Bachère *et al.*, 1995; Gaffney and Bushek, 1996; Tomasso, 1996; Paillard *et al.*, 2004) and increasingly provide tools to help select breeding lines best suited for aquaculture ('domestication'). Gestal *et al.* (2008) provide an excellent and comprehensive review of molecular genetic and genomic studies of the immune responses of bivalve molluscs to many of the microbial diseases described above. Although this is a very positive advance over the past 10 years, genetic selection for open-water productivity (disease resistance/tolerance and growth rates) has raised concerns about the potential impacts on wild

sympatric populations (WWF, 2009). Although evidence of genetic impacts on wild populations from selected stocks spawning in open waters is currently limited or contradictory for bivalves, as genomics and molecular assays advance, the ability to assess accurately the impact of genetic selection on wild populations should become easier. Significant advances in technology and understanding of major production species genomics have already contributed to better understanding of marine bivalve growth and disease resistance (Reid *et al.*, 2003; Huvet *et al.*, 2004; Parry and Pipe, 2004; Hedgecock *et al.*, 2007).

One significant problem associated with disease resistance in bivalves is differentiation between resistant and uninfected bivalves. Neither show clinical signs of disease or, for most cases, readily detectable evidence of the infectious agent (see the section on control and treatment below). The persistence of infectious organisms in resistant (subclinical) bivalve populations is also noteworthy. For example, the period in between epizootics of Malpeque disease (unknown aetiology) averaged 15 years, until the last outbreak in the 1960s. Recent experiments with a historically susceptible population of *C. virginica* have shown that the pathogen is still present, and virulent, in endemic areas, despite no recorded mortalities or clinical signs of the disease in the resident oyster populations for over 25 years (author, unpublished data). This conundrum has hampered effective disease control, such that the disease has now appeared in the last known naive populations of American oysters in Atlantic Canada. As with other bivalve diseases and pathology, it is hoped that biomolecular and genetic analytical tools eventually will shed light on this enigmatic oyster disease.

Cellular defence mechanisms

In clinical infections, microbial agents elicit variable pathogenic effects, dependent on temperature, level of infection and resistance factors. Defence responses range from negligible (e.g. viral gametocytic hypertrophy in American oysters [*C. virginica*]) to focal haemocyte aggregation (e.g. Pacific oyster nocardiosis in Pacific oyster) to systemic haemocyte infiltration, necrosis and mortality (e.g. vibriosis in larval bivalves). Indirect pathogenic effects include tissue irritation, shell or hinge-ligament destruction, which impairs normal respiration and feeding. Direct pathogenic effects include bacterial or fungal exotoxin destruction of cell membranes, organelle displacement or disruption by intracellular proliferation, hypertrophy, lysis or necrosis (Nottage and Birkbeck, 1986; Nottage *et al.*, 1989).

Bivalve defence mechanisms are primarily haemocyte mediated (Tripp, 1960, 1963; Chu, 1988; Feng, 1988; Sparks and Morado, 1988; Bachère *et al.*, 1995); however, there is strong evidence for a humoral component to recognition of foreign particles, including microbial infections (see the section below on biomolecular factors). Haemocytes respond to foreign particles or organisms by: (i) enveloping them to isolate them from surrounding healthy tissue; (ii) destroying them by phagocytosis and/or lysis; (iii) removing them from the host by haemocyte migration across epithelial membranes (diapedesis); (iv) lysis, infiltration and absorption of the foreign body (eukaryote infections); or (v) abscess formation and necrosis of the infected tissue. Some of these responses resemble autolytic, post-mortem changes; however, cells such as multinucleate giant cells are more common in post-mortem necrosis than foreign-body tissue defence (Sparks and Pauley, 1964). Defensive phagocytosis also resembles haemocyte-mediated digestion since a principal component of the food of most filter-feeding bivalves is microbial (Chu, 1988).

Borders of focal lesions may be made up of fusiform or flattened haemocytes (degranulated granulocytic fibroblasts) which create a barrier against the spread of the invasive organism (Auffret, 1988). This is also the primary line of defence along interior tissue surfaces exposed to the exterior by tissue trauma (Sparks, 1985).

Detailed reviews of bivalve haemocyte function, morphology and nomenclature include Elston (1980b), Cheng (1981), Fisher (1986) and Auffret (1988). Morphologically,

hyalinocytes are characterized by a low nucleoplasm–cytoplasm volume ratio, a polymorphic nucleus with one or more distinct nucleoli, numerous mitochondria, endoplasmic reticulum and few cytoplasmic granules ('agranular') (Auffret, 1988). Hyalinocytes frequently are referred to as lymphocytes. Agranular granulocytes have a higher nucleoplasm–cytoplasm ratio than hyalinocytes, a round to ovoid nucleus, but few granular inclusions compared with granular granulocytes. Agranular and granular haemocytes may be referred to simply as granulocytes in the literature. The term 'phagocyte' is used most commonly for granular haemocytes (Auffret, 1988), although agranular haemocytes and hyalinocytes also have phagocytic capabilities. Most bivalves contain a combination of all three cell types; however, scallops and cockles (Pectinidae) lack both granular and agranular haemocytes (Auffret, 1988; Benninger and Le Pennec, 1991). Instead, they have differentiated hyalinocytes, some of which appear to function like granulocytes. See Table 1 in Auffret (1988) for a detailed profile of bivalve haemocytes based on ultrastructural charateristics. A study of bay mussels (*M. edulis*) suffering from haemic neoplasia showed a significant reduction in phagocytic capability (Kent *et al.*, 1989), indicating that dedifferentiation can impair the function of innately phagocytic cells. The heterogeneity of haemocyte morphology and function, however, has prompted recent investigations into the biomolecular properties of morphologically distinct 'types' to determine whether or not they have distinct developmental pathways or represent a continuum. Noël *et al.* (1994) compared five MAbs prepared from *M. trossulus* haemocytes and differentiated four immunostaining groups: Class I MAbs, labelled basophilic granulocytes, Classes II and III, labelled both eosinophilic and basophilic granulocytes, but at different staining intensities, and Class IV MAbs, labelled eosinophilic granulocytes. The authors concluded that this might be evidence of a continuum of antigen production as the 0haemocytes matured.

Nocardiosis in Pacfic oysters (*C. gigas*) (PON) causes intense haemocyte infiltration around the Gram-positive colonies infecting the connective tissue of the digestive gland and mantle (Friedman *et al.*, 1991). In surface tissues, these haemocyte aggregations form greenish-yellow lesions which are readily visible to the naked eye. Similar mantle discoloration may occur with other microbial and eukaryote infections and can be reproduced artificially with talcum powder injections (Pauley and Sparks, 1966). Chronic fibrocyte encapsulation with a defined border is the precursor of collagen encapsulation and subsequent conchiolin deposition (pearl formation) in certain bivalve molluscs (marine and freshwater) (Sparks, 1985).

Many microbial pathogens are intracellular or undergo most of their development within the host cell. If lysed, neighbouring haemocytes engulf the extracellular microbes and cell debris, e.g. in iridoviral infections of the connective tissues in Portugese oyster. Gill necrosis virus (GNV) induces gill connective tissue hypertrophy and intense haemocyte infiltration (Comps, 1988). Viral inclusion bodies in some of the haemocytes (Comps, 1988) indicate that GNV can survive and replicate within the phagocytic cells.

The investigation of phagocytosis of small foreign particles, such as bacteria, has increased significantly over the past 10 years with the production of oyster haemolymph monolayers (Foley and Cheng, 1972), as well as enhanced *in vitro* haemolymph investigation techniques (Burreson *et al.*, 1988; Mortensen, 1995). Extracellular infection by *Vibrio* P1, the causative agent of brown ring disease (BRD) in Manila clams (*T. (= Ruditapes) philippinarum*) (Oubella *et al.*, 1994), elicits a significant increase in total haemocyte count up to 3 days post-intrapallial inoculation. The degree of haemocyte elevation varies with the concentration of bacteria in the inoculum but shows a minimum threshold level of infection required to induce the haemocyte response. Elevated haemocyte levels were maintained for 2 weeks, then dropped, despite continued development of BRD clinical signs. Oubella *et al.* (1994) also noted that the ratio of granulocytes to hyalinocytes

inverted during initial haemocytosis and postulated either a specific haemocyte response to *Vibrio* P1 or a selective cytotoxic effect on the granulocytes. Challenges with *V. pelagius* and *V. anguillarum* did not stimulate haemocytosis. Hyalinocytes and granulocytes of the clam, *T. decussatus*, and mussel, *M. galloprovincialis*, are able to phagocytose *Vibrio* P1, although this capability is consistently greater in the granulocyte fraction of the haemolymph (Villalba *et al.*, 1995). Phagocytosis involved lysosomal fusion with the phagocytic vacuole containing the bacteria, followed by discharge of the resultant residual body.

Haemocytosis is commonly associated with aggregation both *in vivo* and *in vitro* (Feng, 1988). Haemocytes of bivalves demonstrate the capability to form transient (2 h to 3 weeks) as well as permanent aggregations ('clots'), depending on the stimulus, temperature and type of haemocyte involved. Transient forms appear more active in wound repair or physiological stress than in phagocytic aggregations. The role of physical and molecular binding in both transient and more permanent aggregates requires further investigation (Chu, 1988; Feng, 1988; Olafsen, 1988).

Sparks (1985) gives a detailed review of tissue infiltration and repair. The rate of infiltration and repair is temperature dependent; for example, in American oyster, haemocyte activity is negligible below 5°C and sluggish between 5 and 10°C (Stauber, 1950). A similar temperature correlation is found with *in vitro* phagocytosis of *V. vulnificus*, *V. anguillarum* and *B. megaterium* by *C. virginica* and *M. campechiensis* (Roderick and Ulrich, 1984). In addition to phagocytosis, lysosyme levels increased in the extracellular component of the haemolymph in Pacific oysters infected orally with *V. anguillarum*. Parenteral challenges (non-digestive tract), however, did not elicit as distinct an increase (Feng, 1988). Hawkins *et al.* (1993) have also demonstrated a correlation between tidal exposure and haemocyte lysozyme activity in hard-shell clams (*M. mercenaria*). Clams subject to chronic metabolic changes during tidal immersion and demersion showed decreased levels of lysosyme activity and lower numbers of granulocytes compared with control clams.

In contrast, some antibacterial and other haemolymph-mediated defence responses in bivalves appear to be inducible via physical shock, e.g. acute temperature, demersion and/or 'mechanical shock' (Tirard *et al.*, 1995). Initial evidence of such induction has been shown in *M. galloprovincialis*, *O. edulis* and *C. gigas*; however, the exact parameters behind such induction are not yet understood (van der Knapp *et al.*, 1995; Hubert *et al.*, 1996). In addition, immunostimulation against memorized or specific antigen (aquired immunity) rather than non-specific (innate) recognition of non-self has yet to be clearly demonstrated (Chu, 1988).

Biomolecular factors

The 2008 review by Gestal *et al.* provides a comprehensive review of the biomolecular factors involved in bivalve immune response and known correlations to genetic expression. As the advancements made since the last edition of this chapter constitute a chapter in their own right, the reader is recommended to refer to Gestal *et al.* (2008) for more details in this area.

The presence of non-enzymic serum proteins (lectins), which bind to specific carbohydrate groups, is well documented in bivalves and other invertebrates (Bayne, 1983; Russo and Tringali, 1983; Cheng *et al.*, 1984; Flower *et al.*, 1985; Mullainadhan and Renwrantz, 1986; Chu, 1988; Olafsen, 1988; Cooper, 1994) and many act as opsonins, enhancing phagocytic defence mechanisms (Tripp, 1966; Renwrantz and Stahmer, 1983; Olafsen, 1986, 1988). Lysosomal enzymes from bivalve serum, such as β-glucuronidase, have also demonstrated bacteriolytic properties (Cheng *et al.*, 1975; Yoshino and Cheng, 1976; Chu, 1988; Pipe, 1990). Macroglobulins, such as IgM ($\alpha_2$macroglobulin), have been detected in bivalve (as well as gastropod and cephalopod) haemolymph serum (Armstrong and Quigey, 1992), along with IgG (antiagglutinin immunoglobulin G) in blue mussels (*M. edulis*) (Renwrantz and Stahmer, 1983). These macroglobulins protect host proteases

against protease inhibitors, which form a component of the pathogen's defence system.

In vitro observations using luminol-dependent chemiluminescence (LDCL) and nitroblue tetrazolium (NBT) demonstrated the occurrence of oxidative activity in the haemocytes of the mussel, *M. galloprovincialis*, but not the Portugese carpet clam (*R. decussatus*). Both molluscs showed lytic enzyme activity (phosphatases, esterases, proteases and glycosidases) but peroxidase and NADH oxidase activity was detected only in the mussel haemocytes (Villalba *et al.*, 1995). Japanese scallops (*P. yessoensis*) (Nakamura *et al.*, 1985), American oysters (Anderson *et al.*, 1994; Austin and Paynter, 1995) and Pacific and European oysters (Bachère *et al.*, 1991) also show oxidative capabilities. As with Portugese carpet clams, however, hard-shell clams (*M. mercenaria*), soft-shell clams (*M. arenaria*) and cockles show no such ability (Cheng, 1976; Anderson *et al.*, 1994; Lopez *et al.*, 1994).

An interesting host response to *V. tapetis* infection of Manila clams (*T. philippinarum*) and *R. crassostreae* infection of juvenile American oysters is the unusual deposition of laminar conchiolin around the peripheral margin of the shell (Bricelj *et al.*, 1992; Paillard *et al.*, 1994, 2004). A melanin-like pigment forms a component of the conchiolin deposit, which is a more complex response than that of nacrezation (pearl formation) of fungal, metazoan or abiotic tissue irritants (Cheng and Rifkin, 1970; Alderman, 1976). Melanin is a well-documented antimicrobial substance in crustacean defence systems (Brock and Lightner, 1990) but its role in microbial infections of bivalves has yet to be clearly elucidated (Paillard, 1995). The exact cause–effect pathways for similar brown deposits in the nacre layers of American oysters suffering from *Roseobacter* oyster disease, pearl oyster shell deformities and abnormal shell discoloration in coquille St Jacques (*P. maximus*) have been studied (Pass *et al.*, 1987; Bricelj *et al.*, 1992; Larvor *et al.*, 1995; Paillard *et al.*, 1995); however, the biomolecular role in defence function remains to be fully understood (Paillard, 2004).

Despite the wide range of cellular and extracellular components identified in bivalve immune responses, there are still a number of microbial pathogens which elude detection as non-self. For example, rickettsial colonies in the epithelial cells of digestive tubules, ducts and gills rarely stimulate haemocyte recognition, while other infections of the same tissues evoke strong haemocyte infiltration. Recognition proteins, cytolysins and antibacterial proteins have been found in mussels, oysters and shrimps, and reporter genes, under the control of the HSP 70 (heat shock protein) promoter from *Drosophila*, have been used in transfection experiments using embryos and dissociated cells from these shellfish (Cadoret *et al.*, 1994). Results indicate genetic modification to enhance innate resistance factors (Gestal *et al.*, 2008); for example, work on genetic markers in resistant versus non-resistant populations of oysters exposed to the protistan diseases, MSX (*H. nelsoni*) and Dermo (*P. marinus*) shows potential for isolating the genes resposible for inherent, specific disease resistance (Gaffney and Bushek, 1996). In addition, results using the antimicrobial peptide, magainin (extracted from the skin of the frog, *Xenopus laevis*), demonstrated a 94% reduction in the viability of the intrahaemocytic protistan *Bonamia ostreae* of European oysters (Morvan *et al.*, 1994).

Cephalopoda Cuvier, 1797

Wound repair and defence mechanisms in cephalopods have been reviewed by Sparks (1985) and Ford (1992). Wounds induced by experimental insertion of sterilized thread stimulate a similar response in all the cephalopod groups studied. Cuttlefish respond more quickly than squid, but both respond more quickly than octopuses (Jullien, 1928, 1940). Haemocytes infiltrate the wound within 6 h and spread to adjacent connective tissue within 12 h. By 24–96 h, both flattened and normal appearing haemocytes encapsulate and penetrate the fibres of the thread and the lesion develops

into an abscess by day 6. The necrotic contents are encapsulated by flattened haemocytes which, after approximately 2 weeks, elongate. After 4 weeks, the collagenous fibres between the lesion and the epidermis break down and the thread, plus encapsulating haemocytes, is shed. The open wound is sealed via muscular contraction and epidermal mitosis and migration (Polglase *et al.*, 1983; Hanlon and Forsythe, 1990).

Hatchling octopuses (*O. joubini* and *O. briareus*) held in high-density groups (250–300 per 93×31×10 cm tray and 75 per 50×38×12 cm tray, respectively) for biomedical studies demonstrated bacterial infections of skin lesions (Hanlon *et al.*, 1984). The skin lesions developed into epidermal ulcers which spread through the dermal layers to the underlying musculature. Most untreated *O. joubini* died within 4 days of the first sign of infection, indicating a lack of effective defence mechanisms against systemic spread of the bacteria. A similar, but less pronounced, disease progression was reported in *O. briareus*. The species *O. maya* and *O. bimaculoides* were exposed to infected *O. joubini*, but showed no skin lesion development, indicating a possible species-specific susceptibility. The reason for this was postulated as either a difference in epidermal durability or host defence mechanisms against the bacteria and their exotoxins (Hanlon *et al.*, 1984). Demonstration of haemagglutinin (a substance which elicits agglutination of haemocytes) in *O. vulgaris* suggests a possible lectin-like role in immune defence (Russo and Tringali, 1983). Further information on cephalopod lectins was reviewed by Ford (1992).

The phagocytic capacity of the common cuttlefish (*S. officianalis*) against experimentally introduced bacteria was described by Beuerlein *et al.* (2002). Endocytotic cells in the branchial heart complex were studied using an *in vitro* perfusion of bacterial suspensions. The rhogocytes (ovoid cells of the branchial complex) ingested all sizes of tested particles, as did haemocytes of the circulating blood and the adhesive haemocytes in the wall of the branchial heart. Ultrastructural observations further suggested that the triangular mesenchymatic cells were fixed haemocytes that had migrated into the branchial heart tissue and that they were also involved in the defence system of the squid.

Conversely, a very interesting cephalopod–*Vibrio* symbiosis has been described whereby the luminescent bacteria not only inhibit phagocytosis but facilitate development of the luminescent organ of the nocturnal Hawaiian bob-tail squid (*Euprymna scolopes*) (Koropatrick *et al.*, 2007). This complex relationship appears to be mediated by genetic adaptation by the bacteria (Visick *et al.*, 2000). The bacteria provide light disruption camouflage that protects the squid at night, while the squid provides the nutrient basis for bacterial proliferation. Bacterial levels are controlled by diurnal 'purging' of the light organ crypts colonized by the bacteria.

Gastropoda Cuvier, 1797

The best-described defence mechanism against microbial infection of marine gastropods is that against *X. californiensis*, the intracellular bacterium responsible for withering foot syndrome in abalone. Severe metabolic alterations are detected before atrophy of the foot, characteristic of the disease, is manifest. Haemocyanin concentration in the blood, glycogen in the foot muscle and haemocyte abundance all decreased significantly. In addition, abnormal haemocytes were observed in abalone with advanced infections (Friedman, 1996; Shields *et al.*, 1996). Haemocytes were chemotactically active but appeared unable to engulf or destroy foreign particles (Friedman *et al.*, 1999, 2000). The same bacterium is found in healthy abalone with no apparent haemocyte or metabolic response, indicating that evironmental triggers to the host–pathogen relationship are required. This supports the observation that heavy mortalities are correlated to El Niño years with elevated sea temperatures;

however, spread also suggests that previously unexposed ('naive') populations are less tolerant of infections.

Crustacea Pennant, 1777

Cellular defence mechanisms

Pathogenesis and immunity in lobsters, shrimps, prawns, crabs and crayfish are, generally speaking, similar and are discussed together under the heading 'Crustacea'. The initial response to tissue damage (traumatic or due to infection) is haemocyte infiltration. Perforations in the cuticle are sealed by plugging the opening with haemocytes. Subsequent repair involves envelopment of the exposed soft tissues by migrating epidermal cells and melanocytes (Fontaine and Lightner, 1973; Sparks, 1985). If the resulting melanotic 'cyst' is epidermal or cuticular, it is shed at the next moult (Fontaine and Lightner, 1973, 1974; Fontaine *et al.*, 1975; Sparks, 1985).

Experimental intramuscular inoculation of penaeid shrimps with 0.2 ml of a 10% carmine particle in seawater suspension resulted in 100% mortality 30 min post-injection (Fontaine *et al.*, 1975). However, 0.03 ml of a 1.4% carmine–saline solution induced a haemocyte-mediated defence. Within 30 h, both phagocytosed and free carmine particles were accumulated in the heart, gills and at the injection site. Melanocytes encapsulated the carmine-containing haemocytes, becoming evident as brown nodules on the gills and pereiopods by day 4. Diapedesis of the carmine-containing haemocytes takes place across the midgut epithelium of the hepatopancreas for the first 4 days, after which they may also be excreted via the external pore of the antennal gland.

Unlike crustacean defence responses to abiotic foreign materials, microbial infections elicit highly variable tissue responses (Johnson, 1983; Lightner *et al.*, 1992b). In heavy viral infections, there may be cell hypertrophy and lysis, but no apparent haemocyte infiltration or phagocytosis. Differential pathogenicity of morphologically identical viruses is also well documented for different penaeid species (Johnson, 1983; Brock and Lightner, 1990). The fact that wild populations of the same species are affected less severely than their cultured relatives further indicates that some penaeid defence systems may be compromised under culture conditions. This may also be true for lobsters and other crustaceans, which appear more susceptible to microbial infections when held in captivity, prior to live marketing.

Biomolecular factors

The mechanism of immunity and defence responses to microbial infections in shrimps, crabs and crayfish is less well understood than in other invertebrates. Lectin- or enzyme-mediated mechanisms are known to play a role in insect defences and may be involved in crustacean immunity (Söderhall and Unestam, 1979; Bang, 1983; Olafsen, 1988; Adams, 1991). Lightner and Redman (1977) clearly demonstrated the chemical link between phenol oxidases in the circulating haemolymph and the melanization response in penaeid shrimps. A similar reaction is recognized for melanization in response to fungal hyphae (notably of *A. astaci*) in North American crayfish (*Pacisfastacus* spp. and *Procambarus* spp.) (Söderhall and Unestam, 1979). In the more susceptible European crayfish, this melanization response is slow or absent, allowing proliferation of the fungus into the subcuticular tissues, with an estimated 1000-fold greater pathogenic effect than in North American species (Unestam, 1975). The high moleclar weight glycoproteins (β-1,3-glucans) of the fungal hyphae act as a catalyst for the phenol oxidase-mediated melanization (Söderhall and Unestam, 1979).

Antibacterial substances have been found in the freshwater crayfish, *M. rosenbergii*, which have cecropin-like characteristics when challenged with *Escherichia coli* cells (Song *et al.*, 1994). Cecropins are a family of peptide antibiotics found in the haemolymph of certain insects. Astacidae and Cambaridae crayfish also demonstrate distinct variability in lysosyme bacteriocidal capability (Fenouil

et al., 1995). This was linked to increased water temperature and stress from repeated haemolymph sampling. A similar variation in bacteriocidal activity was found in blue crab (*C. sapidus*) in heavily polluted waters off North Carolina. Crabs with bacterial shell disease had a significantly lower level of serum antibacterial activity (0.2%) than crabs without shell disease in non-polluted waters (Noga *et al.*, 1994).

One of the most detailed studies on pathogenesis in a crustacean host is that of *A. viridans* var. *homari* in the lobsters *H. americanus* and *H. vulgaris* (see Stewart, 1984). No mortalities occur at 1°C; however, the bacteria are not shed or killed and may remain viable for at least 250 days. At 3°C, mortalities start after about 180 days and this decreases to 2 days at 20°C. The initial defence reponse is rapid phagocytosis of the bacteria and transportation to the hepatopancreas and cardiac tissues. *A. viridans* var. *homari* evade lysosomal destruction and proliferate using the glycogen and non-protein nitrogen of the haemocyte. The phagocytes are destroyed and release the bacteria into the haemolymph, where they continue to thrive, despite the presence of inducible bactericidins which are effective against a wide range of other bacteria. These bactericidins are adsorbed by both the active and inactive forms of *A. viridans* var. *homari* (Stewart and Zwicker, 1972; Mori and Stewart, 1978). Agglutinins form another effective defence against most bacteria in lobsters, but show no affinity for the surfaces of the virulent gaffkemia bacteria. Interestingly Stewart (unpublished observation in Stewart, 1984) noted that avirulent strains of *A. viridans* var. *homari* were agglutinated and had a negligible capsular layer compared with virulent strains. A precipitin found in lobster haemolymph (Stewart and Foley, 1969) was also ineffective against virulent *A. viridans* var. *homari*, along with three lectins which agglutinated red blood cells and *Brevibacter* sp. but were not adsorbed on to their surfaces. In isolation, the same lectins showed minimal affinity for other bacteria, compared to whole haemolymph (Stewart, 1984). Ironically, it appears that phagocytosis may provide an ideal substrate for proliferation of the gaffkemia bacteria, rather than an effective defence (Patterson and Keith, 1992).

Symbiont-mediated protection

An interesting symbiont-mediated defence mechanism was described by Gil-Turnes *et al.* (1989) where larvae of the shrimp *P. macrodactylus* were found to be unusually resistant to infection by the marine fungus *L. callinectes*. The resistant larvae were coated by an *Alteromonas* sp. that produced 2,3-indolinedione, which inhibited growth of *L. callinectes*. No other symbiont-mediated protection against microbial pathogens of shellfish have been reported.

Echinodermata Klein, 1734

Knowledge of the echinoderm immune response has evolved significantly since the last edition of this chapter, where current knowledge was summarized in the review by Jangoux (1990). A recent review by Smith *et al.* (2006) provides a detailed overview of both the cellular and genetic triggers for regulatory proteins and receptors. The genetic framework for the immune system of the purple sea urchin (*S. purpuratus*) is further described by Hibino *et al.* (2006).

The coelomic fluid in healthy echinoderms is normally sterile. Moreover, viral and bacterial agents inoculated into the coelom of healthy echinoderms are rapidly cleared (Bang and Lemma, 1962; Coffaro, 1978; Wardlaw and Unkles, 1978; Yui and Bayne, 1983). The primary line of defence is the coelomocytes (Smith, 1981; Bang, 1982; Karp and Coffaro, 1982), which phagocytose or encapsulate foreign particles and contain haemolysins, haemagglutinins (Ryoyama, 1973, 1974; Bang, 1982), mucins capable of immobilizing microorganisms (Johnson, 1969), fungicides and antiviral factors (Shimada, 1969; Shimizu, 1971). Coelomocytes known as 'red spherule' cells produce bactericidal naphthoquinone pigments (Johnson, 1969; Wardlaw and Unkles, 1978; Service and Wardlaw, 1985; Wardlaw *et al.*, 1988) and antibacterial

lysozyme (Canicatti and Roch, 1989; Haug *et al.*, 2002). Antibacterial compounds have also been isolated from starfish eggs (Stabili and Pagliara, 1994) and sea urchin seminal plasma (Stabili and Canicatti, 1994).

Molecular studies of sea urchin immune systems have demonstrated a complex complement system with multiple pathways, including several activators of the lectin pathway (Gross *et al.*, 1999, 2000; Pancer, 2000; Smith, 2001; Smith *et al.*, 2001; Shah *et al.*, 2003; Multerer and Smith, 2004).

Diagnostic Methods

Standard techniques recommended for screening commercially important shellfish species for specific pathogens are provided by the American Fisheries Society (Thoesen, 1994) and the Office International des Epizooties (OIE, 2009a).

Non-specific techniques

Tissue squash

Certain microbial infections can be detected in fresh tissue squashes using light microscopy. Virions cannot be seen directly; however, tissue pathology may allow presumptive diagnosis. For example, loss of the velar epithelium can be used for presumptive diagnosis of OVVD (Elston, 1989). Nuclear polyhedrosis viruses (Baculoviridae – NPV) of shrimps and crabs can also be identified presumptively using light microscopy. The spherical or polyhedral intranuclear occlusion bodies fluoresce under ultraviolet light when stained with aqueous 0.1% phloxine (Thurman *et al.*, 1989) and can be distinguished from intracellular organelles, secretory granules or lipid droplets using a 0.1% aqueous solution of malachite green. Brown and Brenn histologic Gram stain (Luna, 1968) also stains NPV more intensely than surrounding tissues.

Endosymbiotic bacteria on the gills of bivalves can be detected by removing gill tissue, washing it in filtered seawater, transferring to 25% seawater and fixing in ethanol:acetic acid (3:1). The tissue is then macerated in 50% acetic acid and a droplet of the resultant suspension is dropped on to a heated (44°C) microscope slide (Thiriot-Quiévreux and Soyer, 1986). The slide is air-dried and stained with Giemsa. Gram stain can also be used on fresh tissue, e.g. penaeid haemolymph. Supplemental procedures (such as electron microscopy, serology or culture), however, are required for specific identification (Lightner, 1983).

Histology

The standard disease-screening technique applied to shellfish is histology (Howard and Smith, 1983; Bell and Lightner, 1988; Bucke, 1989). Although frequently insufficient for species identification of microbial pathogens (especially those with a non-specific host response), histology provides a useful record of infection foci and host response. The host–parasite interaction at the tissue and cellular level is essential for interpreting pathogenic effects. Furthermore, light microscopy is frequently required for selecting optimal tissues for ultrastructural examination.

Standard fixatives used include Davidson's fixative (Shaw and Battle, 1957; Howard and Smith, 1983; Bell and Lightner, 1988; Bucke, 1989), 10% buffered formalin and 1G4F (1 part gluteraldehyde: 4 parts formaldehyde) (Howard and Smith, 1983). Carson's fixative, which is recommended for fixing mollusc tissues in many European laboratories, is similar to 1G4F, but uses paraformaldehyde instead of buffered formaldehyde. Some laboratories use Bouin's fixative; however, the picric acid component of the solution renders it hazardous for long-term storage, transport and disposal. Optimal fixation requires live or moribund specimens, ideally within 12 h of removal from the water to reduce dehydration pathology changes in live specimens. Fixatives such as buffered formaldehyde or Davidson's solution may be used on intact soft tissues removed from the shell. Effective tissue fixation requires a fixative solution to tissue ratio of at least 10:1 by volume.

Shellfish larvae less than 5–10 mm are fixed whole. Bucke (1989) recommends a double-embedding technique where preserved larvae are concentrated into a pellet using centrifugation in molten agar. The agar is allowed to set and then embedded in paraffin. Although the agar takes up some stain (basophilic), it does not affect the staining properties of the larvae. Good results have also been achieved by placing larvae in biopsy bags (VWR Scientific, USA) which are put inside tissue cassettes (M.F. Stephenson, Gulf Fisheries Centre, Moncton, New Brunswick, Canada, 1995, personal communication) for paraffin embedding. Bivalves less than 10–15 mm may be fixed in their shells, after severing the hinge ligament to facilitate fixative penetration of all the soft tissue. Large bivalves are usually removed from the shell and appropriate tissue sections (less than 5 mm thick) taken for fixation. Alternatively, non-gluteraldehyde-based fixatives may be used for larger portions of tissues.

Sections containing calcareous deposits (e.g. pearls or shelled larvae) require decalcification prior to embedding and sectioning. Decalcification can be achieved using ethylenediaminetetracetate (EDTA) (Howard and Smith, 1983) or another chelating agent, which dissolves the calcium carbonate without affecting tissue preservation adversely. Other chelating agents include 16% 4 N formic acid, recommended by Bucke (1989) for controlled decalcification, or other commercial decalcifiers. Decalcified tissues must be rinsed thoroughly in running tap water before dehydrating for paraffin/resin embedding.

Fixation of small crustacean tissues is via injection through the carapace to avoid tissue damage during dissection (Bell and Lightner, 1988). Anatomy diagrams in a number of different invertebrate biology references may be used for other invertebrates, e.g. Hyman (1955), Bullough (1958), Johnson (1980), Bevelander (1988) and Jangoux (1990). Following fixation, tissues are removed for sectioning and embedding.

Stains used routinely for paraffin-embedded tissue sections (Howard and Smith, 1983; Bucke, 1989) include haematoxylin and eosin, which stain nuclei red (except for the diffuse chromatin of mature ovocytes) (C.A. Farley, NOAA-DNR Oxford Laboratory, Oxford, Maryland, USA, 1988, personal communication), along with mucin, cartilage and some Gram-negative bacteria (including rickettsial- and chlamydial-like organisms). Gram's methods are used to detect bacteria and various modifications are recommended, e.g. Brown and Brenn which stains Gram-positive organisms blue (e.g. *Nocardia*, gaffkemia, *Micrococcus*) and Gram-negative organisms red, while tissues appear yellow. The Giemsa method stains *Rickettsia* and *Chlamydia* parasites blue-black, nuclei are blue and other structures are pink or light-blue. Grocott's modified Gomori chromic acid, methenamine silver-nitrate stain (Grocott's methenamine silver – GMS), is recommended for fungi (Bucke, 1989) which stain black (as do Gram-positive bacteria such as *Nocardia* sp. and *Aerococcus* sp.). Mucin stains brown and background tissues stain light green. Farley's Feulgen picromethyl blue stain (Howard and Smith, 1983) is recommended to differentiate DNA from RNA and is useful for viral-like intracellular inclusions. DNA stains red, RNA yellow, connective tissue blue, muscle and cytoplasm are yellow/green. Double-strand DNA and single-strand nucleic acids (including RNA) are differentiated histochemically using acridine orange fluorescence (Gale *et al.*, 1981). Malt periodic acid Schiff (MPAS) is used for PAS-positive reactions with cellulose and starch, notably in fungal tissue infections.

Histocytology

The most common haemolymph examination method is the heart smear, which entails blotting a tissue section through the pericardial cavity on to a slide. Another method, known as the Farley well (after its originator, Mr C.A. Farley, NOAA-DNR Oxford Laboratory, Oxford, Maryland, USA, 1988, personal communication), uses a plastic embedding ring attached to a clean microscope slide by a fabric-coated hairband. This permits removal of haemolymph

from live bivalves and the monitoring of disease progression in individual specimens (Ford and Kanaley, 1988). This technique is also useful for rapid screening of large numbers of bivalves (Burreson *et al.*, 1988). Haemolymph collected by syringe from the adductor muscle sinus is suspended in sterile, filtered, ambient seawater to minimize aggregation of the haemocytes. The syringe needle is removed and the solution placed in the Farley well. Haemocyte adhesion to the microscope slides is enhanced by coating the surfaces with 1% poly-L-lysine. The haemocytes adhere to the glass surface within 30 min (at 10°C), so the plastic ring can be removed and the sterile filtered seawater can be drained. The cells adhering to the slide are fixed and stained as for a blood smear. A modification of this technique uses cannulation to permit repeated haemolymph sampling while minimizing the number of tissue traumas (Jones *et al.*, 1993). Haemolymph samples from live shrimps, crayfish and lobsters are removed from the abdominal sinus, through the mid-dorsal membrane (e.g. for monitoring gaffkemia). Samples from crabs are collected through the articulating membranes at the base of the walking legs.

Electron microscopy

Microorganisms causing histopathology detectable using light microscopy often require higher resolution to permit identification. Transmission electron microscopy (TEM) is used for ultrathin tissue sections (<1 µm), negative stain preparations or microbial suspensions. The negative stain technique is more rapid than tissue section examination but provides no information on the host–pathogen interface, and high levels of infection (10^6–10^7 particles/ml) are necessary to ensure detection of the agent (Hetrick, 1989; Hayat and Miller, 1990).

Culture of microorganisms

Since the previous edition of this chapter, no successful progress has been made in the development of a multigeneration or self-replicating molluscan or shrimp cell line. Rinkevich (2005) provides an excellent review of progress made up to the late 1990s, along with some interesting speculation on the application of new cellular, genomic and proteomic tools to this problem. Fundamentally, however, application to the field of marine invertebrate pathogen isolation has been superseded by molecular detection tools (as described above). However, for sensitivity/specificity validation of molecular detection tools, a cellular isolation medium remains a significant need.

The bulk of the work up to the 1990s focused on marine molluscs and freshwater/marine penaeid shrimp (Mialhe *et al.*, 1988a; Chen, 1990; Rosenthal and Diamant, 1990; Kleinschuster and Swink, 1992). Explants of cardiac tissue from molluscs have been kept alive for up to 117 days (Tripp, 1963) and cell lines of heart tissue from *C. gigas* and *O. edulis* have been subcultured for 4 days. Monolayer production from heart and mantle cells from *C. virginica* was enhanced by use of poly-L-lysine and fibronectin, along with a thin layer of 0.5% low melting point agarose to inhibit cell migration (Faisal *et al.*, 1993). Monolayers of lymphoid tissues and ovary from *P. monodon*, *P. japonicus* and *P. penicillatus* survive at least 20 days (Chen, 1990). Insect cell lines have also been used (Lo *et al.*, 1988) but use of non-aquatic or vertebrate cell lines increases the problem of differentiation between true aquatic pathogens and cell-line contaminants (Hill *et al.*, 1986). No mitosis has yet been observed in aquatic invertebrate cell monolayers.

Standard culture media and techniques used for bacteriology of shellfish are reviewed in detail by Elston (1989). Vibrios are most commonly identified using thiosulfate citrate bile salts sucrose agar (TCBS agar), where sucrose fermenting strains turn the medium yellow. The TCBS agar is supplemented with 1% (w/v) sodium chloride (NaCl) or filtered seawater which has been diluted to provide 1% (w/v) additional NaCl. Samples are incubated at the ambient water temperature of the host and examined within 24–48 h for discoloration of the green culture medium and/or formation of yellow colonies (Elston, 1989).

Elston (1989) provides a detailed list of media routinely used to monitor shellfish-holding facilities:

1. Marine 2216E agar (MA, Difco) – an enriched medium suited for rapidly growing, non-fastidious, marine bacteria such as *Vibrio* spp. *Vibrio* colonies are Gram-negative, non-aerogenic, oxidase positive and contain motile rods.
2. Trypticase (=tryptone)-soy agar (TSA, Difco) – supplemented with 1% (w/v) NaCl or diluted seawater is an enriched medium for non-fastidious bacteria, e.g. *Vibrio* spp. and ulcerative bacteraemia of cephalopods. TSA or trypticase-soy broth (TSB) is also used to grow Gram-positive bacteria, such as *A. viridans* (var.) *homari* (Stewart *et al.*, 1966).
3. Seawater cytophaga agar (SWCA) – favours slow-growing colonies, such as: *Cytophaga*-like bacteria (CLB) which cause hinge-ligament disease in juvenile bivalves; *Flexibacter* spp. and *Flavobacter* spp. (filamentous yellow-pigmented bacteria). Identification of CLB on SWCA is by rhizoid colony morphology, gliding motility and variable cell lengths (3 to >100 µm) grown from excised ligament tissue.
4. Brain–heart infusion agar (BHIA) – used for acid-fast, Gram-positive bacteria such as *Nocardia* spp., identified by their beaded and branched mycelia. The same medium is used to confirm *A. viridans* var. *homari* (gaffkemia) following presumptive culture in phenylethylalcohol (PEA) broth. BHIA inhibits Gram-negative proliferation and is presumed positive for *A. viridans* var. *homari* when the medium colour changes from purple to yellow (acid production) within 4 days at 28°C. Positive PEA broth media are inoculated on to PEA agar plates and colonies of Gram-positive cocci inoculated on to the BHIA for confirmation of β-haemolysis and catalase-negative properties (Stewart *et al.*, 1966). Gram-positive *Streptococcus* spp. are differentiated by their short chains of cocci, rather than the tetrad formations of *Aerococcus* spp. (Stewart *et al.*, 1966; Pappalardo and Boemare, 1982). An indirect immunofluorescent antibody detection technique (IFTA) has also been developed for *A. viridans* var. *homari* (Marks *et al.*, 1992) and is described under the section below on immunological antibodies and assays.
5. Löwenstein-Jensen agar medium was used to isolate acid-fast *Mycobacterium* spp. Runyan Group II (slow-growing *Mycobacterium* sp. with carotenoid pigmentation) from the freshwater prawn *M. rosenbergii* (Brock *et al.*, 1986a). Following decontamination of tissues[7] containing the characterisic brown/black nodules (Vestal, 1978, in Elston, 1989), incubation (in light or dark) at 30–37°C produced orange pigmented colonies within 14 days.

Standard culture media used for shellfish mycology are reviewed in Hatai (1989). Glucose–yeast extract agar (GY agar) prepared with distilled water is recommended for culture of freshwater fungi, e.g. *Aphanomyces* spp. and *Saprolegnia* spp. (Hatai and Egusa, 1979). Saprolegnid colonies are easily distinguished from Aphanomycid colonies by hyphal diameter (30–40 µm in *Saprolegnia* and 7–9 µm in *Aphanomyces*). Other culture media recommended for *A. astaci* are RGY agar (riverwater–glucose–yeast extract agar) (Alderman, 1982), Difco Bacto-peptone + glucose + basal medium 2 (Unestam, 1965) and crayfish blood + fish meat extract agar (Alderman and Polglase, 1986).

O. implexa can be cultured from small pieces of shell decalcified in 5% EDTA (see the section on histology). The decalcified shell is immersed in a 0.5% sodium hypochlorite–seawater solution for 30 s and incubated in filtered seawater or a yeast extract–peptone medium containing antibiotics. Fungi grown from the shell fragments are subcultured on yeast extract–peptone medium. Raghukumar and Lande (1988) found that zoospore production was enhanced when the yeast extract–peptone medium was supplemented with either 0.01% cholesterol or 0.01% lecithin.

Bian *et al.* (1979) recommend culture of *Atkinsiella* spp., *Haliphthoros* spp. and *Lagenidium* spp. on PYGS (peptone–yeast–glucose–seawater) agar. Proportions vary slightly from the recipe of Fuller *et al.* (1964)

for *L. callinectes* and Lightner (1988e) for *L. callinectes*, *Sirolpidium* spp. and *Haliphthoros* spp.. Following incubation at 20°C for 2–10 days, sporulation is induced by transferring the mycelia produced to sterile filtered seawater.

Higher fungi, such as *F. solani*, are cultured on Sabouraud's dextrose agar (SDA) (Lightner, 1988d), Bacto-mycobiotic agar (Hatai *et al.*, 1978), thioglycollate medium (Sindermann, 1988b), potato dextrose agar (PDA) or potato sucrose agar (PSA) (Lightner, 1988d; Hatai, 1989). Irrespective of medium, *Fusarium* cultures should be incubated for at least 4 days at 25°C to allow formation of the conidia. QPX (Quahaug parasite X), which has chytrid-like features, was also cultured using PDA (Whyte *et al.*, 1994). The addition of the antibiotics penicillin G or streptomycin sulfate is recommended to inhibit bacterial growth in all these media.

Pathogen-specific techniques

Since the last edition of this section, when immunoassays and molecular diagnostic tools were in the developmental stage with limited application to routine pathogen detection and identification (Schill *et al.*, 1989; Lightner *et al.*, 1992a; Mialhe *et al.*, 1992; Bachère *et al.*, 1995), biomolecular assays have become the primary tool for this purpose. The numbers of DNA- and RNA-based probes applied to certification of pathogen-free stocks have grown exponentially and are now routinely applied to transfer/trade of live aquatic organisms, as well as certain products (OIE, 2009a); however, many remain to be validated properly for sensitivity/specificity. Pathogen-specific techniques fall into two categories: (i) immunoreagents and assays; and (ii) nucleic acid probes and detection assays (Mialhe *et al.*, 1992).

Immunological antibodies and assays

In the 1980s and 1990s, extensive progress was made with the isolation and purification of microbial antigens (Cousin *et al.*, 1990; Maes and Paillard, 1990), which facilitated development of polyclonal and monoclonal antibodies that bound specifically with them (Elston *et al.*, 1981; Mialhe *et al.*, 1988b; Le Gall and Mialhe, 1992). More recently, however, biomolecular technology has overtaken immunoassays as the preferred analytical technique for rapid, pathogen-specific screening and identification (see below). Despite this, however, immunoassays continue to be developed and applied to valuable microbial pathogen research.

Polyclonal antibodies (PAbs) are produced from a generalized immune response and specificity is enhanced by using highly purified antigen or absorbing antisera with host antigens to remove cross-reacting antibodies. PAb recognizes a broad spectrum of antigens which occur in different developmental stages of a pathogen or in closely related species (Choi *et al.*, 1991). Inherent difficulties with PAbs are consistency in production and variability of attachment of covalent labels (Hetrick, 1989; Cousin *et al.*, 1990; Choi *et al.*, 1991; Mialhe *et al.*, 1992). Monoclonal antibodies (MAbs) are more specific, honing in on a single antigenic protein, and have been developed for the giant rickettsia infecting the scallop, *P. maximus*, and *Vibrio* P1 in Manila clam (*T. philippinarum*) (Maes and Paillard, 1990; Le Gall and Mialhe, 1992; Mialhe *et al.*, 1992). MAbs have also been produced for neoplastic haemocytes in mussels (Noël *et al.*, 1991) and appear capable of distinguishing between different normal haemocytes of oysters and mussels (Morvan *et al.*, 1991; Noël *et al.*, 1991). Mialhe *et al.* (1988b) describe the protocols for producing MAbs for bivalve pathogens and state that the major obstacle to MAb production is isolation and purification of the pathogen. Immunization using intact infected cells for initial sensitization(s) followed by a terminal booster with small quantities of purified antigen has shown promise in overcoming this obstacle, and some antibody production has been achieved with whole-cell immunization (Mialhe *et al.*, 1992).

Production of PAbs or MAbs against specific pathogens is required to produce direct and indirect immunofluorescent antibody techniques. Infected cells or tissues

(smears or sections) are identified by antibody binding to pathogen antigen. Intracellular pathogens are rendered accessible by acetone treatment to increase cell membrane permeability (Mialhe *et al.*, 1992). An indirect immunofluorescent antibody technique (IFAT) has been developed to screen lobsters for gaffkemia (Marks *et al.*, 1992). Cross-reactivity of the antibody for *A. viridans* var. *homari* with *Staphylococcus aureus* was overcome by papain (an enzyme used to cleave immunoglobulins) treatment of the haemolymph smears prior to IFA staining. Papain also enhances the fluorescence of positive slides (Marks *et al.*, 1992). Two avirulent strains of *A. viridans* var. *homari* (ATCC 10400 and 37R) were IFAT positive, but showed narrower bands of fluorescence than virulent strains. The avirulent strains ATCC 88A and 88B were IFAT negative. IFAT is particularly useful for detecting early infections where phagocytosed bacteria are lodged in the heart and hepatopancreas (Marks *et al.*, 1992). Indirect fluorescent antibodies have also been developed to detect baculoviral midgut-gland necrosis (BMN) in kuruma shrimp (*P. japonicus*) (Sano *et al.*, 1984) and polyclonal antibodies were used to distinguish non-pathogenic *Vibrio* species from *Vibrio* P1, the causative agent of 'brown ring disease' of Manila clams (*T. philippinarum*) (Castro *et al.*, 1995). Slide agglutination and indirect immunofluorescence microscopy revealed strong cross-reactivity of the PAb to *Vibrio* P1; however, a dot–blot immunoenzymatic assay showed greatest sensitivity and had the advantage over the other two techniques of not requiring microorganism isolation or tissue sections (Castro *et al.*, 1995).

A second type of immunoassay is the enzyme-linked immunosorbent assay (ELISA), a relatively quick, quantifiable method for assessing infections using MAb or PAb. To date, no MAb-ELISA has been developed for microbial pathogens of shellfish and the PAb-mediated ELISA produced for penaeid baculovirus (Lewis, 1986) is of limited use because it requires large amounts of antibody to coat the solid phase of the assay (also a problem for MAb-ELISA). As pathogen purification and antibody production are refined, however, ELISA technology may become more practical (Mialhe *et al.*, 1992; Bachère *et al.*, 1995).

Biomolecular assays

Biomolecular assays have been refined to a level of technical precision and scope over the past 10 years that prohibits a realistic review for the revision of this chapter. Specific assays and detailed protocols are provided in the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2009a), along with reference laboratories responsible for validating the sensitivity and specificity of the assays.

Control and Treatment

Transmission

Emphasis is placed currently on control of disease transmission between stocks and populations, as well as between host species. Control of the spread of contagious disease agents, via transfer of live shellfish from one facility, area or country to another, is contingent upon the availability of accurate diagnostic tools for screening for specific disease agents. Since many microbial disease agents cannot be detected at subclinical infection levels, infected hosts have been able to transmit many diseases between populations and countries (De Voe, 1992).

Control of non-contagious, opportunistic microbial disease agents in closed-circulation holding systems may be managed with chemotherapeutants (discussed below) or by modified husbandry practices (Elston, 1990). Foremost in preventing a disease problem in a shellfish-holding facility or open-water site is the health of introduced stocks: (i) availability of accurate health histories from their source; (ii) records of movements prior to introduction; and (iii) regular monitoring of the system for potential pathogens.

Due to a paucity of treatments for most shellfish diseases and the location of much

intensive shellfish culture in open water, or flow-through facilities, disease control is commonly undertaken by: (i) avoidance of the introduction of contagious pathogens into specific pathogen-free facilities, areas or countries; and (ii) management strategies to circumvent mortalities and maintain commercial production in areas where a pathogen has become established.

Introductions, transfers and trade

The standard protocols used since the 1970s to reduce the risk of pathogen transfer with the introduction and transfer of live aquatic animals are those developed by the International Council of the Exploration of the Seas (ICES) in cooperation with the European Inland Freshwater Advisory Council (EIFAC), which have recently been updated (ICES, 1994, 2004). Standards applied to specific aquatic animal diseases including those of molluscs and crustaceans are set by the World Organization for Animal Health Office of Epizootics (Office International des Epizooties – OIE) in support of the World Trade Organization's Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) (WTO, 1994). The OIE standards are revised regularly to reflect the appearance of 'new' diseases and the development of new diagnostic techniques (OIE, 2009a).

Circumvention of mortalities

There are no cases of successful eradication of a shellfish disease agent from open-water areas. Korringa (1976b) described successful management of shell disease in European oysters (*O. edulis*) due to the fungus *O. implexa*. Oyster shells were removed regularly ('cleaned') from Dutch oyster beds, which were then re-seeded with seedstock selected from unaffected broodstock. Although this did not eradicate the problem, it did reduce shell deformation to a commercially acceptable level.

Circumvention of mass mortalities of penaeid shrimp due to luminescent bacteria has been achieved by: (i) increased monitoring of the influent water to prevent entry of luminescent bacteria (Baticados *et al.*, 1990); or (ii) postponing production of postlarvae during periods when luminescent bacteria are detected (Tonguthai, 1992). Although this does not prevent the disease, losses are reduced by continual assessment of environmental conditions. When the level of luminescent bacteria reaches a threshold of tolerance, production is postponed.

Most other examples of circumvention of chronic shellfish diseases involve protistan parasites (Andrews and Ray, 1988; Bower, 1988; Ford and Haskin, 1988; van Banning, 1988); however, the strategies employed may be applicable to microbial diseases.

An alternative circumvention strategy is to harvest the shellfish before peak mortalities. Although this reduces the immediate commercial impact in enzootic areas, both susceptible and potentially resistant oysters are removed from the spawning population, thus slowing down or obstructing development of disease resistance. Resistant strains can be developed experimentally (Ford and Haskin, 1995); however, the effort required is usually long term and costly in comparison with closing a select area to exploitation and allowing natural resistance to build up. Resistant stock are a valuable seed source for enzootic growing areas, and stock which survive in a multi-pathogen environment are invaluable (no matter how few).

An extreme circumvention strategy, which has been used repeatedly to maintain shellfish industries destroyed by disease, is replacement of the susceptible species by another (not necessarily indigenous) resistant species. The most common bivalve example is the Pacific oyster, which has been spread from Japan throughout shellfish-culturing countries since the early 1900s (Andrews, 1980). Subsequent field and laboratory experiments with Pacific oyster on the west coast of Canada, as well as DNA sequencing studies, however, have shown that it may carry protistan disease organisms which can be transmitted to other oyster species (Bower *et al.*, 1994b; Burreson, 1996).

Husbandry practices

The principal method of disease control in hatcheries is destruction of the infected shellfish and disinfection of the tanks and equipment they contacted (Elston, 1990, 1993). In some cases, however, early detection and removal of individuals with clinical signs may control pathogen proliferation or enhance effective chemotherapeutant treatment of remaining stocks (see the section below on treatment and protection).

All prophylactic measures involve risk:benefit examination of production goals. Intense production is only economically preferable to less intense production where mortalities are proportionately equal to or lower than in the former. Production at densities greater than found in the natural environment are clearly linked to the following stress factors (Elston, 1984):

- mechanical damage due to overcrowding (cuticle or shell damage)
- insufficient nutrients, oxygen, or other metabolic requirements
- excessive waste production (nutrient for biofouling)
- biofouling of the stocks and/or pipeline equipment, inhibiting optimum waterflow
- temperature, salinity or photoperiod changes too rapid for adequate acclimatization.

These factors enhance significantly the susceptibility to opportunistic microbial infection. Tolerance thresholds for stocking density, rates of temperature, salinity or photoperiod change, nutrient concentrations, etc., vary between species and have to be determined for individual facilities and sites, to minimize disease losses (Prieur and Carval, 1979; Tomasso, 1996). Korringa (1976a,b) provides detailed husbandry information for oysters, Lutz (1980) for mussels, Shumway (1991) for scallops, Manzi and Castagna (1989) for clams and Morse *et al.* (1984) for Pacific molluscs in general. Korringa (1976c), New (1982), McVey (1983) and Fast and Lester (1992) provide husbandry information for shrimp species. Fisher *et al.* (1978) and Fisher (1988a) provide nutritional information in relation to bacterial shell disease in lobsters. Many hatcheries disinfect influent water to control levels of epibiont fouling. For example, initial 1 µm filtration followed by UV treatment plus 0.45 µm filtration provides the water reservoir used for the Galveston method of penaeid larviculture (Smith *et al.*, 1992). Measures recommended for reducing or eliminating disease outbreaks in molluscan hatcheries are outlined by Elston (1984, 1990, 1993) and include regular monitoring of influent water, algal cultures and broodstock for bacterial or fungal build-up (see the section on diagnostic methods).

In addition to screening for opportunistic pathogens, obligate pathogens, which can be detected using rapid diagnostic techniques, can also be managed within a hatchery environment. For example, *B. penaei* is reduced or eliminated from penaeid shrimp hatcheries (Chen *et al.*, 1992) by:

1. Selection of nauplii from broodstock females which are not passing polyhedrin occlusion bodies (POBs) in their faeces or have POB-negative hepatopancreas tissue squashes;
2. Ensuring minimal exposure of female shrimps to eggs and nauplii, e.g. by equipment changes, footbaths, alternative water sources for broodstock and larval-holding facilities;
3. Reduction of between-tank transfers;
4. Elimination of BP from contaminated equipment using an alkaline disinfectant; and
5. Use of seawater free of pesticides, chlorinated hydrocarbons or heavy metals which may enhance BP pathogenicity to exposed larvae.

Quarantine and disinfection

Quarantine is commonly used to control disease spread. Types of quarantine range from high technology facilities, with automated fail-safe containment and treatment equipment, to tanks with disinfected effluent. The first documented quarantine for a shellfish disease was for Malpeque disease in American oysters from Prince Edward

Island (PEI), Canada, in 1915 and 1916. Although the cause of the mortalities was not known, oyster transplants out of Malpeque Bay were prohibited (geographic quarantine). The disease did not reappear until the 1930s, when apparently healthy Malpeque oysters were transplanted to other bays around PEI (Needler and Logie, 1947). The entire province was, therefore, placed under an oyster export quarantine and the disease was contained until the 1950s, when 'bootleg' oysters were shipped to New Brunswick for processing. The disease agent is believed to have infected New Brunswick oyster stocks via shells and meats discarded outside processing plants. Susceptible New Brunswick oysters were replaced by Malpeque resistant PEI oysters (Drinnan and England, 1965), thus transforming southen Gulf of St Lawrence oysters into a homogeneous population of subclinical carriers of an unidentified disease agent!

Successful quarantining of a holding facility relies on effective disinfection of effluent and materials leaving the facility, either through eradication of a specific type of pathogen or all living organisms (sterilization). Disposable equipment may be autoclaved, immersed in an iodophor, chlorine or sodium hydroxide solution. Reusable equipment can be washed with the same solutions (ensuring the chemical compatibility with the surface being treated).

Treatment of effluent water requires removal of suspended solids, via filtration through sand or cartridge filters (to 5–1 μm), to enhance subsequent sterilization with ozone, ultraviolet (UV) light or chlorine solutions which bind to organic compounds. Several authors provide detailed technical information on the safe treatment of effluents and equipment (Blogoslawski and Alleman, 1979; Townshend, 1980; Elson, 1983; Elston, 1990; Fast and Lester, 1992; Boyd, 1996). It is worth noting, however, that the residual oxidant component of these disinfectants and the residual compounds they form with seawater are highly toxic to shellfish. Disinfected water must be neutralized by aeration or sunlight exposure (for chlorine) or activated charcoal filters (for chlorine and ozone residual compounds). Chlorine neutralization using sodium thiosulfate is not recommended for flow-through water, since it is also highly toxic to shellfish larvae and large quantities are required (Boyd, 1996).

Regular monitoring of treated effluent is necessary to ensure effective disinfection, since small numbers of microbial agents may survive. No standard concentration of chemical disinfectant should be applied without a preliminary investigation of the disinfectant demand of the water to be treated. The efficacy of disinfectants, such as chlorine, varies with pH and the amount of ions present to form combined residuals (as opposed to free residuals). Combined residuals are less effective disinfectants than free residuals and a higher concentration of chlorine is required to compensate for this factor in the water (Boyd, 1996).

Filtration (0.8 μm mesh) of disinfected effluent water is necessary to prevent transmission of IHHNV of penaeid shrimps for at least 30 days post-exposure (Lotz *et al.*, 1990), since IHHNV can survive ozone and air-drying treatments. Ozonation is also ineffective against the zoospore stages of the fungal-like protistan *Labyrinthuloides haliotidis* (Bower, 1989; Bower *et al.*, 1989). Several authors recommend serial disinfection with ozone or chlorine followed by UV light, especially for quarantine systems requiring total eradication of disease agents (Blogoslawski and Alleman, 1979; Townshend, 1980). Verification of effuent sterility is done by bioassay or routine bacteriology and mycology, as described in the section on diagnostic methods.

Treatment and protection

Chemotherapy

Chemotheraprutants have long been used in finfish aquaculture (Meyer and Schnick, 1989; Michel and Alderman, 1992); however, there is increasing awareness of the risks associated with indiscriminant or continuous application. These risks include drug-resistant pathogens, overdosage and

impacts on the environment, consumer (health and market perception) and aquaculturist (handling and economics). Moreover, inceased restrictions on aquaculture imports between countries with non-complementary drug and chemical regulations (Meyer, 1992; Plumb, 1992; Schnick, 1992; Alderman *et al.*, 1994) have led to increased interest in developing alternative disease controls and improving husbandry techniques in order to reduce the need for chemical prophylaxis.

Effective use of chemotherapeutants is restricted to systems where concentrations of shellfish, water volume/quality/flow rate and drug concentration can be calculated. Chemotherapeutant treatment of diseases such as *V. tapetis* of Manila clams (*T. philippinarum*) has been investigated, and nitrofuran antibiotics were found to be effective under experimental conditions. Administration on a commercial scale in open water, however, is considered problematic, due to documented mutagen and carcinogen effects in vertebrates (Noël *et al.*, 1990, 1992). Alderman (1992) discusses the use of chemotherapeutants in molluscan aquaculture, including treatment of *Vibrio*-P1. Flumequine (a group 4 quinolone) is cited as being used in extensive growing conditions; however, there appears to be no further information on its use to control brown ring disease. Alderman *et al.* (1994) note strong environmental questions with respect to accumulation of this artifical antibiotic in bottom sediments. Similar problems with the development of resistance to oxalinic acid (4-quinolone) and furazolidone by sediment bacteria (Nygaard *et al.*, 1992; Samuelsen *et al.*, 1992) are also under investigation.

In intense culture systems, disease outbreaks can occur with devastating rapidity, making effective treatment impossible after the clinical disease signs have been detected. Thus, most chemotherapeutants used in hatcheries and holding facilities are administered prophylactically and on a continuous basis. Chronic use of chemicals (mainly antibiotics) in bivalve hatcheries has been studied extensively (Hidu and Tubiash, 1963; Ray, 1965; Calabrese and Davis, 1967; D'Agostino, 1975; Tubiash, 1975; Le Pennec and Prieur, 1977; Bower, 1989) and their efficacy questioned (Le Pennec and Prieur, 1977; Alderman, 1992). The most commonly used antibiotics in bivalve culture were penicillin–streptomycin or chloramphenicol combinations (Walne, 1958; Bayne, 1965; Minaur, 1969; Elston, 1990; Alderman, 1992); however, subsequent studies have shown these to have unreliable efficacy in seawater (Le Pennec and Prieur, 1977) or under certain hatchery conditions (Alderman, 1992). Cycloheximide is an antifungal and antiprotistan (Ray, 1965; Bower, 1989); however, it is toxic to the algal food of bivalves and ineffective against bacteria and the infective stages of certain protistans. Furthermore, cycloheximide-resistant forms of the abalone parasite *Labyrinthomorpha haliotis* appeared within three treatments (Bower, 1989). Addition of chloramphenicol (chloromycetin) to the penicillin–streptomycin combination increased bacteriocidal efficacy over a short period (48 h) (Millar and Scott, 1967), even though chloramphenicol is antagonistic to penicillin action (Singleton and Sainsbury, 1991). Chloramphenicol appeared promising for several bivalve species; however, the concentrations which inhibited larval growth, or were toxic to the larvae, were inconsistent (Le Pennec and Prieur, 1977). Notably, chloramphenicol is now widely recognized as inducing rapid resistance in Gram-negative bacteria and its use in aquaculture is banned in several European countries, as well as Canada (Alderman *et al.*, 1994). Since the microbial organisms in any hatchery comprise an array of viruses, bacteria and fungi, the use of a single antibiotic is less effective than a combination and accelerates the development of resistant pathogens (Nygaard *et al.*, 1992; Plumb, 1992; Alderman *et al.*, 1994).

Of 14 antibacterial substances tested on a number of different cephalopod species by Forsythe *et al.* (1990), only nifurpirinol demonstrated efficacy. Repeated bath treatments with nifurpirinol resulted in 73% recovery, with skin lesions healing after 7–10 days of 3–10 min baths in 1.0–2.0 mg/l (Hanlon *et al.*, 1984). Initial formation of a smooth epidermis over ulcerated areas

occurred 7–10 days following the start of treatment. Regeneration of the underlying musculature and functional chromatophores occurred 27–74 days later, depending on the severity of the lesion at the start of the treatment (Hanlon *et al.*, 1984). Other treatments were ineffective or showed minimal inhibition. Octopuses required 1–2 months to heal and had to be held in isolation (octopuses held in groups failed to recover) (Hanlon and Forsythe, 1990). One species, *O. briareus*, had an apparent toxic reaction to treatment after 32 days. A pseudomonad and *A. anitratus* on a single octopus (*O. dofleini*) at the National Aquarium in Baltimore were eradicated following 28 days of tetracycline treatment administered via injection into its live prey (Stoskopf *et al.*, 1987, cited in Hanlon and Forsythe, 1990). Laboratory reared *O. bimaculoides* and *O. maya* suffering from limb loss due to infection by *V. carchariae* via frozen shrimp were treated successfully with chloramphenicol injected into live or frozen shrimp. However, recurrent infections occurred 1–3 weeks post-treatment and required additional treatments (Hanlon and Forsythe, 1990). The same authors report positive results using oral or intramuscular injection of chloramphenicol or gentomycin, against *V. splendidus*, *V. pelagius* (Biogroup II) and *P. stutzeri* in the haemolymph of captive European cuttlefish (*S. officianalis*), especially if administered prior to development of the dorsal haematoma.

Shell pitting and erosion of chitinolytic bacteria in the crayfish *M. rosenbergii* was inhibited by a 1 h bath in 10 ppm oxolinic acid. Exoskeletal lesions were lost at the next moult (El-Gamal *et al.*, 1986). Treatment of other non-penaeid crustaceans affected by chitinolytic bacteria has not been reported.

The use of drugs to control bacterial proliferation in intense shrimp culture is more widespread than in bivalve or other crustacean culture (Bell and Lightner, 1992). The scale and intensity of shrimp aquaculture in some countries, as well as the ease of administration to ponds, raceways and tanks, and in shrimp feed, make continuous prophylactic chemotherapeutant treatment feasible (Baticados *et al.*, 1990; Shariff *et al.*, 1992). Oxytetracycline has been widely used since the mid-1980s to treat vibriosis (black splint disease) in shrimp culture (Takahashi *et al.*, 1985b), as well as Texas pond mortality syndrome (Bell and Lightner, 1992; Lightner *et al.*, 1992b). However, the efficacy of this and other treatments against *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. has dropped significantly due to indiscriminate overuse (Nash *et al.*, 1992; Ruangpan and Kitao, 1992). Of 19 chemotherapeutants (including oxytetracycline and furazolidone) tested against five species of *Vibrio* from dying *P. monodon* (black tiger shrimp), a relatively new sulfur drug (VP_{2647}) appeared the most effective (Plumb, 1992). Similar results have been found in hatchery-reared *P. indicus* infected by *V. campbellii* where, out of 11 antibiotic treatments, only chloramphenicol proved effective (Hameed and Rao, 1994). Subsequent mass mortalities of *P. monodon* larvae at a shellfish hatchery showed the presence of an O/129-resistant *V. harveyi* buiding up in the hatchery tanks, whereas the same species of *Vibrio* in influent and outside water sources was susceptible to O/129 treatment (Karunasager *et al.*, 1994). Further testing of the O/129-resistant *V. harveyi* revealed concurrent resistance to streptomycin, chloramphenicol and cotrimoxazole. Similar results have been documented with *V. cholerae* isolated from cholera patients in India (Ramamurthy *et al.*, 1992).

Treatment of fungal infections of shrimp, such as larval mycoses, has also been relatively prophylactic. The compound most commonly used (an agricultural herbicide, Triluralin) has little effect on the systemic hyphae, but is lethal to the infective zoospore stage (Bell and Lightner, 1992). Another agricultural herbicide, Triforine, inhibits normal phagocytosis in oyster (*C. virginica*) haemocytes (Alvarez and Friedl, 1992).

Vaccination

Only four vaccination studies have been reported for shellfish. In the 1960s, Evans

et al. (1968) induced bactericidin activity in the spiny lobster (*P. argus*) using a vaccine which consisted of killed bacteria cells. McKay and Jenkin (1969) also reported vaccine-induced immunity to bacterial challenge in the Australian crayfish (*Parachaeraps bicarinatus*). Since these trials, two vaccines have been developed against *A. viridans* var. *homari* (gaffkemia) in American lobsters (*H. americanus*) (Stewart and Zwicker, 1974; Keith *et al.*, 1992; Patterson and Keith, 1992) and a *Vibrio* sp. NU-1 in kuruma prawn (*P. japonicus*) (Itami *et al.*, 1989, 1991, 1992).

The vaccines against gaffkemia were developed from killed *A. viridans* var. *homari* cells, with and without supplemental vancomycin (a complex glycopeptide antibiotic active against Gram-positive bacteria). Both were effective under laboratory conditions and vaccinated lobsters showed enhanced survival in field trials. Vaccine administered by immersion was less protective than vaccination by injection (Keith *et al.*, 1992; Patterson and Keith, 1992).

The vaccine against *Vibrio* sp. strain NU-1 uses formalin-killed bacteria (Itami *et al.*, 1989). Injection, immersion and spray exposure to the vaccine all showed progressive protection against live *Vibrio* sp. injections. Mortality declined 6–10 days post-challenge and was half that of non-vaccinated controls. The development of an effective immersion, spray or microencapsulated feed vaccine appears promising for intense shrimp aquaculture (Itami *et al.*, 1989, 1991, 1992).

Suggestions for Further Study

With the exponential increase in trade of shellfish and shellfish products in fresh or frozen state, the potential for microbial pathogens to spread concomitantly has been well demonstrated. This has provided an equally exponential scope for microbial pathogen research, most notably in the field of molecular biology, and has provided immense advantages and improvements in the control of known diseases; however, it also comes with rapid application in an environment where we still have limited knowledge of what 'microbially' is 'out there'. Validation is essential to provide meaningful interpretation of positive and negative results. Without well-established pathogen specificity, these tools risk inaccurate host specificity. In addition, as with human mutagenic pathogens, such as the orthomyxoviruses, a huge research challenge will be the prediction of impending changes in microbial pathogenicity. Although daunting, understanding the role of changing environmental conditions, as well as the host–parasite interface, is essential. Shellfish microbial pathogen research has benefited immensely over the past 10–15 years from collaboration with human and terrestrial pathogen researchers. The next generation of research appears set to take these advances further into a multidisciplinary approach encompassing biotic and abiotic factors in both the host and non-host environment. The multidisciplinary approach will be essential as global changes effect change at the minutest microbial and host physiological levels.

Acknowledgements

Many micrographs were prepared from bivalve histopathology slides generously donated to the author by C.A. Farley. Original electron micrographs were produced at the Electron Microscopy Unit, University of New Brunswick. Special thanks are also due to D. Noël, V. Boulo, J.P. Cadoret, J.-C. Veyrunes and M. Bergoin, Comité National de la Recherche Scientifique, France; R.A. Elston, Battelle Research Laboratory; D.V. Lightner, University of Arizona; G. Messick, National Oceanographic and Atmospheric Administration – Department of Natural Resources, Oxford Laboratory; C. Friedman, University of California; S. Boonyaratpalin, Thailand and the Southeast Asian Fisheries Development Centre, Fish Health Unit, Tigbauan Marine Station, Philippines (C. Pitogo, E. Cruz, R. Fernandez, L. de la Penna), who kindly provided micrographs or tissue samples.

Notes

[1]International Standards Organization: ISO/IEC 17025:2005 'General requirements for the competence of testing and calibration laboratories'.
[2]Office International des Épizooties.
[3]Type A nuclear inclusions are amorphous or particulate and condensed in rounded masses which do not stain like nucleoli. Nuclear reaction is total and proceeds to complete degeneration. All basophilic chromatin is marginated. Type B nuclear inclusions are localized in certain areas of the nucleus. The nucleoplasm may not be altered significantly and shows no margination. Type B inclusions do not destroy the nucleus.
[4]Unpublished information reported, as required, to the OIE (http://www.oie.int/wahis/ public.php?page=single_report&pop=1& reportid=8265, accessed 12 June 2010).
[5]Distinct from infectious hypodermal and haematopoietic necrosis virus (IHHNV), described separately.
[6]OIE (2007) World Animal Health Information Database (WAHIS) (http://www.oie.int).
[7]Decontamination is required to remove faster growing bacteria which will overgrow the slower growing *Mycobacterium*.

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21 Genomics of Fish and Shellfish Microbial Pathogens

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Genomics of Fish Bacterial Pathogens

Genome sequence information is a valuable source of information for studying pathogenesis, virulence, immunogenicity, therapeutic targets and diagnosis of fish pathogenic bacteria. In addition, this information increases our understanding of the host–pathogen interaction for developing prevention methods for fish and shellfish infectious diseases in aquaculture. In the microbe genome sequence database of NCBI as of 12 January 2010 (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), the number of completed genome sequences of bacteria including those that are pathogenic and non-pathogenic is 986, those in assembly stage are 1149 and those that are unfinished (under various stages in genome projects) are 1186. Some of these are fish pathogenic bacteria (Table 21.1). Four species, *Aeromonas salmonicida* subsp. *salmonicida* (Reith *et al.*, 2008), *Aliivibrio salmonicida* (Hjerde *et al.*, 2008), *Flavobacterium psychrophilum* (Duchaud *et al.*, 2007) and *Renibacterium salmoninarum* (Wiens *et al.*, 2008), were isolated from fish, and four species, *A. hydrophila* (Seshadri *et al.*, 2006), *Mycobacterium marinum* (Stinear *et al.*, 2008), *Vibrio harveyi* (Genbank accession No. NC_009783, NC_009784) and *V. splendidus* (GenBank accession No. NC_011744, NC_011753), were not taken from fish. Moreover, four species, *Edwardsiella ictaruli*, *E. tarda*, *Lactococcus garvieae* and *Streptococcus iniae*, are currently being completed, while two species, *V. anguillarum* (Rodkhum *et al.*, 2006a,b) and *Photobacterium damselae* subsp. *piscicida* (Naka *et al.*, 2005), have been partially sequenced (Table 21.1). Recently, the whole genome sequence of *E. tarda* has been completed (Wang *et al.*, 2009). These bacteria possess a single chromosome of 3.76 Mbp containing 3486 predicted protein-coding sequences. The following section presents the completed genome sequence of four fish pathogenic bacteria, *F. psychrophilum* (Duchaud *et al.*, 2007), *A. salmonicida* subsp. *salmonicida* (Reith *et al.*, 2008), *Al. salmonicida* (Hjerde *et al.*, 2008) and *R. salmoninarum* (Duchaud *et al.*, 2007), and the partial genome sequence of two fish pathogenic bacteria, *P. damselae* subsp. *piscicida* (Naka *et al.*, 2005) and *V. anguillarum* (Rodkhum *et al.*, 2006a,b).

Aeromonas salmonicida subsp. salmonicida

A. salmonicida subsp. *salmonicida* is a Gram-negative bacterium and the causative agent of furunculosis in salmonid fish. Furunculosis has been recognized as an important disease in wild and farmed fish. The genome size of *A. salmonicida* subsp. *salmonicida* strain A449 is 4.7 Mbp encoding

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Viral, Bacterial and Fungal Infections, 2nd Edition (eds P.T.K. Woo and D.W. Bruno)

Table 21.1. Genome sequences of fish pathogenic bacteria.

Species	Genome size (Mbp)	Proteins	References or Groups
Genome sequences of fish isolated bacteria (completed)			
Flavobacterium psychrophilum	2.86	2,432	Duchaud *et al*. (2007)
Aeromonas salmonicida subsp. *salmonicida*	4.70	4,388	Reith *et al*. (2008)
Aliivibrio salmonicida	3.33 and 1.21	4,175	Hjerde *et al*. (2008)
Renibacterium salmoninarum	3.16	3,507	Wiens *et al*. (2008)
Edwardsiella tarda	3.76	3,486	Wang *et al*. (2009)
Genome sequences of non-fish isolated bacteria (completed)			
Aeromonas hydrophila	4.74	4,122	Seshadri *et al*. (2006)
Mycobacterium marinum	6.64	5,423	Stinear *et al*. (2008)
Vibrio harveyi	3.76 and 2.20	5,944	NC_009783, NC_009784
Vibrio splendidus	3.30 and 1.68	4,431	NC_011744, NC_011753
Genome sequences of fish pathogenic bacteria (in progress)			
Edwardsiella ictaruli	ND		University of Oklahoma
Lactococcus garvieae	ND		Kitasato University
Streptococcus iniae	ND		Baylor College of Medicine
Genome sequences of fish pathogenic bacteria (partial)			
Photobacterium damselae subsp. *piscicida*	ND		Naka *et al*. (2005)
Vibrio anguillarum	ND		Rodkhum *et al*. (2006a,b)

4388 genes (Reith *et al*., 2008). Many of the genes are potentially virulence related, including virulence factor secretion system (type II and III secretion systems), adhesion (flagella and pilus), toxins (aerolysin, haemolysin, RTX toxin, endotoxins), secreted enzymes (proteases, nucleases, chitinases, glycerophospholipid cholesterol acyltransferase, esterase, collagenase), iron acquisition (siderophore synthesis and uptake, haem receptors) and quorum sensing-related proteins (Table 21.2). Based on the genomic sequence, Nash *et al*. (2006) developed a DNA microarray containing 2024 genes for *A. salmonicida* subsp. *salmonicida*. They studied the variation of different isolates of the bacteria and found that many known virulence genes were common to all strains tested. Some of these virulence-related genes were knocked down and their pathogenicity against salmonid fish was confirmed. Dacanay *et al*. (2006) demonstrated that the type III secretion system (TTSS) was essential for virulence of *A. salmonicida* subsp. *salmonicida* by using single gene knockout mutant strains. Recently, Boyd *et al*. (2008) examined the virulence of the pili of *A. salmonicida* subsp. *salmonicida*. The bacteria have no visible pili but their genome contains genes for three type IV pilus systems including Tap, Flp and mannose-sensitive haemagglutinin pilus (MSHA). They found that the Tap contributed moderately to the virulence to Atlantic salmon (*Salmo salar*) but Flp made little or no contribution to virulence. Boyd *et al*. suggested that the *A. salmonicida* subsp. *salmonicida* type VI pili were not absolutely required for virulence in Atlantic salmon. Interestingly, Masada *et al*. (2002) reported that the *A. salmonicida* subsp. *salmonicida* type IV pilin (Tap) was required for virulence in rainbow trout (*Oncorhynchus mykiss*). From these studies, it was hypothesized that type IV pilus system in *A. salmonicida* subsp. *salmonicida* had a role in host–pathogen interaction.

Aliivibrio salmonicida

Al. salmonicida (formerly *V. salmonicida*) is a Gram-negative bacterium and the causative agent of coldwater vibriosis in Atlantic

Table 21.2. Identified putative virulence factors in *Aeromonas salmonicida* subsp. *salmonicida* strain A449 by genome analysis.

Secretion system	Secreted enzymes
Type II secretion system	Serine protease
Type III secretion system (TTSS)	Zinc metalloprotease
Type VI secretion system	Metalloprotease
Aop H effector	Elastase
Aop effector	Collagenase
Aex effector	Glycerophospholipid cholesterol acyltransferase
Ati 2 putative effector	Phospholipase A1
Putative TTSS effector	Phospholipase C
Adhesion	Extracellular nucleases
Lateral flagella	Amylases
Polar flagellas	Chitinases
Type I pilus	Pullulanase
Tap type IV pilus	Iron acquisition
Flp type IV pilus	Siderophore synthesis and uptake
Msh type IV pilus	Siderophore receptor
Toxins	Haem receptor
Aerolysin	Haem uptake protein
Haemolysin	Quorum sensing
RTX toxin	*N*-acyl homoserine lactone synthase
Cytolytic δ-endotoxin	Quorum sensing regulon activator
	AI-2 synthase
	Quorum sensing phosphorelay protein
	Quorum sensing response regulator

salmon, rainbow trout and Atlantic cod (*Gadus morhua*). Similar to the characteristics of family Vibrionaceae, the *Al. salmonicida* strain LFI1238 genome also consists of two chromosomes. The size of the two chromosomes is 3.3 Mbp (chr I) and 1.2 Mbp (chr II) encoding 3070 and 1105 protein coding genes, respectively (Hjerde *et al.*, 2008). Hjerde *et al.* (2008) found several potential virulence factors in the genome, including secretion systems (type I, II and VI), type IV pilus, haemolysins, iron acquisition systems, etc. (Table 21.3). In some *Vibrio* species, haemolysin, particularly the thermostable direct haemolysin (TDH), is known as one of the most important virulence factors. Interestingly, there are five putative haemolysin genes in *Al. salmonicida* genome, although none of the five genes are homologous to TDH (Hjerde *et al.*, 2008). Recently, five different haemolysin genes have also been found in *V. anguillarum* (Rodkhum *et al.*, 2006a). Two of the genes, *VAH2* and *VAH5*, were homologous to those found in the *Al. salmonicida* genome. In the case of *V. anguillarum*, these haemolysin genes are indicated to be two of the virulence factors in rainbow trout. There is a good possibility that the five putative haemolysin genes of *Al. salmonicida* are involved in virulence, but it is necessary to carry out further experiments to confirm this. By using the genome information of *Al. salmonicida*, it is possible to identify or characterize virulence factors and host restrictions.

Flavobacterium psychrophilum

F. psychrophilum is a Gram-negative bacterium and responsible for the coldwater disease, causing considerable economic losses in salmonid fish and ayu aquaculture worldwide. The genome of *F. psychrophilum* strain JIP02/86 (ATCC49511) consists of a 2.9 Mbp circular chromosome with 2432 protein coding genes (Duchaud *et al.*, 2007). Duchaud *et al.* (2007) found several

Table 21.3. Identified putative virulence factors in *Aliivibrio salmonicida* strain LFI1238 by genome analysis.

Secretion system	Iron acquisition
Type I secretion system	Siderophore synthesis and uptake
Type II secretion system	Siderophore receptor
Type VI secretion system	Haem receptor
Adhesion	Haem uptake protein
Polar flagellas	Toxins
Msh type IV pilus	Haemolysin (VAH2)
Flp type IV pilus	Haemolysin (VAH5)
LPS and cell capsule	Haemolysin
LPS biosynthesis systems	Haemolysin (hlyIII)
	Haemolysin-type calcium-binding protein
	Serine protease
	Peptidase

putative virulence factor genes such as toxins (proteases, haemolysin, thiol-activated family of pore-forming toxin), adhesions and secretion systems (Sec-independent transport systems, PorT and PorR). In the case of putative toxins, there are several different types of proteases, including seven genes for metalloprotease, two genes for metallopeptidase and two genes for serine endopeptidase. Interestingly, they have found only a truncated form of the collagenase gene, which is known as a virulence factor in several pathogenic bacteria including *F. psychrophilum* (Duchaud *et al.*, 2007). Duchaud *et al.* (2007) examined this unexpected result using PCR and sequencing of 23 different isolates of *F. psychrophilum*. They revealed that ten of the 23 strains had the truncated gene, all of which were isolated from rainbow trout. These results suggested that collagenase was not a virulence factor in rainbow trout. They also found the homologue of *V. anguillarum* haemolysin VAH5 in the genome of *F. psychrophilum*, as well as the homologue of the pore-forming toxin thiol-activated cytolysin family (TACY). Such toxins appear to be involved in the pathogenicity of *F. psychrophilum*.

Renibacterium salmoninarum

R. salmoninarum is a Gram-positive bacterium that causes bacterial kidney disease (BKD) in salmonid fish. The genome of *R. salmoninarum* strain ATCC33209 is a 3.2 Mbp circular chromosome encoding 3507 protein-coding genes (Wiens *et al.*, 2008). Wiens *et al.* (2008) reported several virulence-related factors found in the genome, including the haem acquisition systems, capsular synthesis, secretion systems, major soluble antigen (MSA), proteases and haemolysins. Interestingly, there are 68 antibiotic resistance- related genes, including a variety of multidrug transporters, several types of beta lactamase, tetracycline resistance (TetA, TetB and TetP) and macrolide-resistance factors (RlmA, SpoU, MefA, PvsC and KsgA). The MSA is known to be specific to *R. salmoninarum* and it is duplicated in the bacteria's genome. Moreover, some strains have a third copy of the MSA and their virulence is higher than the strains possessing only two copies of MSA gene. Two haemolysins and three haemolysin candidate genes were likewise found.

Photobacterium damselae subsp. piscicida

P. damselae subsp. *piscicida* (formerly *Pasteurella piscicida*), of the family Vibrionaceae, is the causative agent of pasteurellosis in fish. Since 1969, this disease has economically damaged yellowtail (*Seriola quinqueradiata*) production in Japan. Furthermore, pasteurellosis has also damaged the culture of gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) in Europe, striped bass (*Morone saxatilis*) in the USA and snakehead (*Channa argus*), cobia (*Rachycentron canadum*) and hybrid striped bass in Taiwan (Daly and Aoki, see Chapter 17, this volume). Several virulence factors have been reported for these bacteria (Daly and Aoki, see Chapter 17), such as iron acquisition system (Magariños *et al.*, 1994), cell surface capsule (Magariños *et al.*, 1996; Arijo *et al.*, 1998), extracellular products (Nakai *et al.*, 1992), haemolysin

(Magariños *et al.*, 1992) and phospholipase (Naka *et al.*, 2007). By partial genome analysis, 2055 protein-coding genes, including several virulence-related genes containing colonization factors (37 genes), exotoxin (five genes) and lipopolysaccharide biosynthesis-related molecules (six genes) were identified (Naka *et al.*, 2005). Among the colonization factors, 27, three and four genes have homology with polar flagellar-related genes, capsule biosynthesis genes and others (accessory colonization factors, SOD, MshA biogeneis and haem receptors), respectively (Naka *et al.*, 2005). With exotoxin genes, two different types of haemolysins, phospholipase, lysophospholipase and hyaluronidase were found. Extracellular products of *P. damselae* subsp. *piscicida* have virulence activity against mouse and rainbow trout and it is thought that phospholipase and haemolysin are involved because they have been detected in the extracellular products. Naka *et al.* (2007) confirmed that phospholipase of *P. damselae* subsp. *piscicida* had haemolytic activity and found the sensitivities of fish and mammal erythrocytes to phospholipase were very different. Phospholipase showed strong haemolytic activities for fish [yellowtail, carp (*Cyprinus carpio*), rainbow trout, red sea bream (*Pagrus major*), sea bass (*Lateolabrax japonicus*) and tilapia (*Oreochromis niloticus*)] erythrocytes but little or no haemolytic activities for mammalian (rabbit, horse and sheep) erythrocytes. However, the haemolytic activity of the phospholipase was detected in all mammalian erythrocytes with the addition of lecithin. These results indicate that the phospholipase is a lecithin-dependent haemolysin (LDH) against mammalian erythrocytes and a direct haemolysin for fish erythrocytes. The different sensitivities between mammalian and fish erythrocytes to phospholipase might be due to differences in the relative amounts of phospholipids (e.g. phosphatidylcholine, phosphatidylethanol amine, phosphatidylserine and phosphatidylinositol) in the membrane of erythrocytes. Other studies have described results similar to those reported here. *V. mimicus* haemolysin (*phl* gene) has little or no ability to lyse mammalian erythrocytes, although it does lyse fish erythrocyte (Lee *et al.*, 2002). *V. parahaemolyticus* thermolabile haemolysin (*tlh* gene) (Taniguchi *et al.*, 1985), which has phospholipase A activity, has high identities with *V. harveyi* and *V. mimicus* and shows LDH activity (Schmiel and Miller, 1999). The amino acid sequences of all these haemolysins from *Vibrio* spp. are similar to the amino acid sequence of *P. damselae* subsp. *piscicida* phospholipase (Taniguchi *et al.*, 1985; Schmiel and Miller, 1999; Lee *et al.*, 2002).

Vibrio anguillarum

V. anguillarum is the aetiological agent of vibriosis or haemorrhagic septicaemia affecting marine and freshwater fish and shellfish. The *V. anguillarum* genome is composed of two chromosomes similar to other Vibrionaceae (Okada *et al.*, 2005). Several virulence factors have been identified in this species, including iron uptake system, polar flagella, metalloproteases, major surface antigens and haemolysins, by individual cloning experiments (Rodkhum *et al.*, 2006b). Rodkhum *et al.* (2006b) identified 2826 genes by partial genome analysis. Among these, 40 putative virulence-related genes were identified, 36 of which were novel in *V. anguillarum*, including a repeat in toxin (RTX) gene cluster, haemolysin gene, enterobactin gene, protease genes, lipopolysaccharide biosynthesis genes, capsule biosynthesis gene, flagellar genes and pilus genes. The RTX gene operons contain a total of five open reading frames (ORFs) arranged in two operons, rtxAC and rtxBD (Rodkhum *et al.*, 2006b). These operons had RTX toxin gene (*rtxA*) and RTX toxin transporter gene (*rtxB*), and RTX toxin activating protein gene (*rtxC*) and RTX toxin transporter gene (*rtxD*), respectively (Li *et al.*, 2008). The gene organization of this RTX gene cluster was highly similar to the RTX gene cluster of *V. cholerae* O1 biovar E1 Tor str. N16961 (Heidelberg *et al.*, 2000) and *V. vulnificus* YJ016 (Ruby *et al.*, 2005). Sixteen genes encoding the components, which might mediate adherence and colonization to the host cells,

were identified. Several capsule and cell surface component genes that were related to virulence in other bacterial pathogens were found, including the virulence gene, vibriobactin-specific 2,3-dihydroxybenzoate-AMP ligase (*angE*) (Liu *et al.*, 2004). This gene was proven to have a redundant function with *angE* gene on virulence plasmid pJM1 of *V. anguillarum* 775 (Alice *et al.*, 2005). Haemolysin has been suspected of being an important molecule involved in haemorrhagic septicaemia, which is a major sign of fish vibriosis. Some types of haemolysin in *V. anguillarum* have been published and some properties of haemolysin have been verified. Five different types of putative haemolysin genes have been identified and characterized, all of which show high similarity to haemolysin genes in other *Vibrio* species, such as *V. cholerae* O1 biovar El Tor (Heidelberg *et al.*, 2000), *V. parahaemolyticus* (Taniguchi *et al.*, 1990; Makino *et al.*, 2003) and *V. vulnificus* (Chen *et al.*, 2003). The role of these novel haemolysin genes in the virulence of *V. anguillarum* was further investigated and compared with the role of haemolysin genes in other *Vibrio* species, and it was shown that each haemolysin gene contributed to the virulence of *V. anguillarum* in juvenile rainbow trout.

Genomics of Fish and Shellfish Viral Pathogens

Mortalities in cultured fish and shellfish due to infectious viral disease have been a serious problem worldwide for several decades. The major viral-causing diseases are listed in Table 21.4. Whole genome analysis of disease-causing viruses is very important to elucidate and understand the mechanism of virus infection into the host animals. Genomics research for fish and shellfish virus began in the mid-1980s, when researchers discovered a small-sized genome from an RNA virus. Currently, 41 whole genome sequences from a total of 20 fish and shellfish viruses (almost half the species in Table 21.4) have already been published in the GenBank database. These genome sizes vary from 3.1 kb to 307.3 kb (Table 21.5), are linear DNA or RNA, except for that of white spot syndrome virus (WSSV), which is circular double-stranded DNA. The genome of RNA virus encoding two to nine ORFs is small in general (Table 21.6). *Birnaviridae* (e.g. infectious pancreatic necrosis virus and yellowtail ascites virus of the family) and *Nodaviridae* (e.g. red-spotted grouper nervous necrosis virus, *Epinephelus tauvina* nervous necrosis virus, striped jack nervous necrosis virus and *Macrobacterium rosenbergii* nodavirus) have two chromosomes (segment A/B or RNA1/2) and infectious salmon anaemia virus, or isavirus, has nine chromosomes (segments 1~9), encoding a few ORFs within the small size of each segment. On the other hand, the DNA virus genome is much larger in size (>102.6 kb). The WSSV Taiwanese strain (WSSV-TW) has the largest size of all genomes (307.3 kb) among the known infectious virus in fish or shellfish (Table 21.5). The genome structure of *Iridoviridae* [e.g. lymphocystis disease virus (LCDV) and red sea bream iridovirus (RSIV)] could be circularly permuted and terminally redundant (Darai *et al.*, 1985; Tidona and Darai, 1997). The succeeding sections describe the genomics as well as the proteomics of viral haemorrhagic septicaemia virus (VHSV), koi herpesvirus (KHV) and WSSV, which have caused most mass damage to aquaculture.

Viral haemorrhagic septicaemia virus (VHSV)

VHSV, which causes one of the devastating diseases of salmonid and marine fish, is a non-segmented, negative single-strand RNA virus of the genus *Novirhabdovirus*, family *Rhabdoviridae*. The whole genome sequence of VHSV has been determined from eight strains (Table 21.5). The size of the whole genome comprises 10,845~11,182 bp, encoding six proteins: the nucleoprotein or nucleocapsid protein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), the non-virion protein (NV) and the viral RNA polymerase (L) (Schütze *et al.*, 1999). These genes are arranged in the order 3′-N-P-M-G-NV-L-5′. Nucleotide and

Table 21.4. Fish and shellfish infectious virus.

Virus name	Genus	Family	Host animal
Fish			
1 Infectious haematopoietic necrosis virus (IHNV)	Novirhabdovirus	Rhabdoviridae	Salmonids
2 Viral haemorrhagic septicaemia virus (VHSV)	Novirhabdovirus	Rhabdoviridae	Salmonids, Japanese flounder
3 Spring viraemia of carp virus (SVCV)	Vesiculovirus	Rhabdoviridae	Carp
4 Perch rhabdovirus	Vesiculovirus	Rhabdoviridae	Perch
5 Pike fry rhabdovirus	Vesiculovirus	Rhabdoviridae	Pike
6 Infectious pancreatic necrosis virus (IPNV)	Aquabirnavirus	Birnaviridae	Salmonids
7 Yellowtail ascites virus (YAV)	Aquabirnavirus	Birnaviridae	Yellowtail, marine fish
8 Epizootic haematopoietic necrosis virus (EHNV)	Ranavirus	Iridoviridae	Redfin perch, rainbow trout, frog
9 Lymphocystis disease virus (LCDV)	Lymphocystivirus	Iridoviridae	Flounder, sea bream, rockfish
10 Red sea bream iridovirus (RSIV)	Megalocytivirus	Iridoviridae	Red sea bream, Murray cod
11 Singapore grouper iridovirus (SGIV)	Ranavirus	Iridoviridae	Grouper
12 White sturgeon iridovirus (WSIV)	Unclassified	Iridoviridae	White sturgeon
13 Carp pox herpesvirus (CyHV-1)	Ictalurivirus	Alloherpesviridae	Carp
14 Haematopoietic necrosis herpesvirus of goldfish (CyHV-2)	Ictalurivirus	Alloherpesviridae	Goldfish
15 Koi herpesvirus/cyprinid herpesvirus 3 (KHV, CyHV-3)	Ictalurivirus	Alloherpesviridae	Carp
16 Ictalurid herpesvirus 1 (CCHV)	Ictalurivirus	Alloherpesviridae	Channel catfish
17 Flounder herpesvirus (FHV)	Ictalurivirus	Alloherpesviridae	Japanese flounder
18 Herpesvirus salmonis (SalHV-1)	Ictalurivirus	Alloherpesviridae	Salmonids
19 *Oncorhynchus masou virus* (OMV, SalHV-2)	Ictalurivirus	Alloherpesviridae	Salmonids
20 Carp nephritis and gill necrosis virus	Unclassified	Alloherpesviridae	Common carp
21 Red-spotted grouper nervous necrosis virus (RGNNV)	Betanodavirus	Nodaviridae	Red-spotted grouper
22 *Epinephelus tauvina* nervous necrosis virus (ETNNV)	Betanodavirus	Nodaviridae	Greasy grouper
23 Striped jack nervous necrosis virus (SJNNV)	Betanodavirus	Nodaviridae	Striped jack
24 Chum salmon virus (CSV)	Aquabirnavirus	Reoviridae	Salmonds, catfish, bivalves
25 Grass carp rhabdovirus	Aquareovirus	Reoviridae	Grass carp
26 Infectious salmon anaemia virus (ISAV)	Isavirus	Orthomyxoviridae	Salmonids, Atlantic cod
27 Erythrocytic inclusion body syndrome virus (EIBSV)	Unclassified		Salmonids
Shellfish			
1 Baculovirus penaei (BP, tetrahedral baculovirus)	Nucleopolyhedro virus	Baculoviridae	Penaeid shrimps
2 Penaeus monodon-type baculovirus (MBV, spherical baculovirosis)	Nucleopolyhedro virus	Baculoviridae	Penaeid shrimps
3 Baculoviral midgut gland necrosis virus (BMNV)	Unclassified	Baculoviridae	Penaeid shrimps

(*Continued*)

Table 21.4. *Continued*

Virus name	Genus	Family	Host animal
Shellfish			
4 White spot syndrome virus (WSSV)	Whispovirus	Nimaviridae	Penaeid shrimps
5 Yellow head virus (YHV)	Okavirus	Roniviridae	Penaeid shrimps
6 Taura syndrome virus (TSV)	Cripavirus	Discistroviridae	Penaeid shrimps
7 Infectious hypodermal and haematopoietic necrosis virus (IHHNV)	Brevidensovirus	Parvoviridae	Penaeid shrimps
8 Hepatopancreatic parvovirus (HPV)	Unclassified	Parvoviridae	Penaeid shrimps
9 Spawner-isolated mortality virus	Unclassified	Unclassified	Penaeid shrimps, crayfish
10 Mourilyan virus	Unclassified	Bunyaviridae	Penaeid shrimps
11 Infectious myonecrosis virus (IMNV)	Unclassified	Totiviridae	Penaeid shrimps
12 Extra small virus (XSV)	Nodavirus	Nodaviridae	Penaeid shrimps, aquatic insect
13 *Macrobrachium rosenbergii* nodavirus (MrNV)	Unclassified	Nodaviridae	Penaeid shrimps
14 Ostreid herpesvirus type 1 virus (OsHV-1, Pacific oyster herpesvirus)	Ostreavirus	Malacoherpes viridae	Pacific oyster
15 Haemocyte infection virus	Irido-like viruses		Pacific oyster, pearl oyster
16 Gill necrosis virus (GNV)	Irido-like viruses		Pacific oyster, pearl oyster

deduced amino acid sequences revealed significant homology to corresponding sequences in the VHSV strains: Cod Ulcus, Hededam, 14-58 and 96-43 (Betts and Stone, 2000). Of the VHSV proteins, the G-protein is a good example of a molecule that has been applied successfully as DNA vaccine for the rainbow trout (Lorenzen *et al.*, 1998, 2009). The G-protein is especially capable of inducing protective immunity, having been tested, together with the N-protein, as a DNA vaccine for salmonid fish (Lorenzen *et al.*, 1998), as well as a DNA vaccine for infectious haematopoietic necrosis virus, which is under the same genus *Novirhabdovirus* (Corbeil *et al.*, 1999, 2000). The DNA vaccine of VHSV G-protein is also effective in inducing the immune response of Japanese flounder, giving a high survival rate of >93% (Byon *et al.*, 2005).

Koi herpesvirus (KHV)

KHV (alternatively termed cyprinid herpesvirus type-3, CyHV3) has been responsible for outbreaks resulting in high mortalities in the common carp (*C. carpio*) and its variety, the koi carp, both in fish farms and in the natural environment worldwide (McGeoch *et al.*, 2006). KHV classified within the *Alloherpesviridae* family, has the longest known genome among the species of the *Herpesvirales* order, with a double-stranded DNA of about 295 kb, containing 22 kb terminal direct repeat (Hutoran *et al.*, 2005; Waltzek *et al.*, 2005; Aoki *et al.*, 2007). The whole genome sequences of three KHV strains from Japan (strain TUMST1), the USA (strain KHV-U) and Israel (strain KHV-I) have been determined (Table 21.5). From these sequences, 15 KHV genes have been found to have clear homologues with the distantly related channel catfish virus (icutalurid herpesvirus type-1, CyHV1), indicating that the fish herpesvirus is considerably more divergent than the mammalian herpesvirus (Aoki *et al.*, 2007). These genome sequences are interpreted as having arisen from a wild-type parent encoding 156 unique protein-coding genes, eight of which are duplicated in the terminal repeat. Thirty-one kinds of proteins identified in KHV-J genome are listed in Table 21.6. Interestingly, the

Table 21.5. Completed genome sequences of fish and shellfish viruses.

Virus name	Genus	Family	Accession no.	Genome shape (strain/isolation)	Genome type	Length (bp)	Total genome size (bp)	References
Fish virus								
1 Koi herpesvirus (KHV, CyHV-3)	Unclassified	Alloherpesviridae	NC_009127, DQ657948	Complete genome (strain KHV-U, USA)	dsDNA, linear		295,146	Aoki *et al.* (2007)
			AP008984	Complete genome (strain TUMST1, Japan)	dsDNA, linear		295,271	Aoki *et al.* (2007)
			DQ177346	Complete genome (strain KHV-I, Israel)	dsDNA, linear		295,138	Aoki *et al.* (2007)
2 Ictalurid herpesvirus 1 (CCHV)	Ictalurivirus	Alloherpesviridae	NC_001493, M75136	Complete genome (strain Auburn 1, ATCC:VR-665)	dsDNA, linear		134,226	Davison (1992)
3 Lymphocystis disease virus (LCDV)	Lymphocystivirus	Iridoviridae	NC_001824, L63545	Complete genome (LCDV-1)	dsDNA, linear*		102,653	Tidona and Darai (1997)
			NC_005902, AY380826	Complete genome (isolate China, LCDV-C)	dsDNA, linear*		186,250	Zhang *et al.* (2004)
4 Red sea bream iridovirus (RSIV)	Megalocytivirus	Iridoviridae	BD143114	Complete genome	dsDNA, linear*		112,414	Nakajima and Kurita (2005)
5 Singapore grouper iridovirus (SGIV)	Ranavirus	Iridoviridae	AY521625	Complete genome	dsDNA, linear		140,131	Song *et al.* (2004)
6 Infectious pancreatic necrosis virus (IPNV)	Aquabirnavirus	Birnaviridae	NC_001915	Segment A	dsRNA, linear	3,097	5,881	Duncan and Dobos (1986)
			NC_001916	Segment B	dsRNA, linear	2,784		Duncan *et al.* (1991)
			AY283780	Segment A (strain AM-98)	dsRNA, linear	3,088	3,088	Zhang and Suzuki (2004)
7 Yellowtail ascites virus (YAV), marine birna virus (MABV)	Aquabirnavirus	Birnaviridae	NC_004168	Segment A (strain Y-6)	dsRNA, linear	2,976	5,711	Suzuki *et al.* (1998)
			NC_004176	Segment B (strain Y-6)	dsRNA, linear	2,735		Zhang and Suzuki (2004)
8 Infectious haematopoitic necrosis virus (IHNV)	Novirhabdovirus	Rhabdoviridae	NC_001652, L40883	Complete genome (strain WRAC)	ssRNA, linear		11,131	Morzunov *et al.* (1995)

(*Continued*)

Table 21.5. *Continued*

Virus name	Genus	Family	Accession no.	Genome shape (strain/isolation)	Genome type	Length (bp)	Total genome size (bp)	References
Fish virus								
9 Viral haemorrhagic septicaemia virus (VHSV)	Novirhabdovirus	Rhabdoviridae	NC_000855, Y18263	Complete genome (strain Fi 13)	ssRNA, linear		11,158	Schutze *et al.* (1999)
			AB490792	Complete genome (strain JF00Ehi1)	ssRNA, linear		11,182	Unpublished
			EU481506	Complete genome (strain FA281107)	ssRNA, linear		11,065	Unpublished
			Z93414	Complete genome (strain Cod Ulcus)	ssRNA, linear		10,845	Stone *et al.* (1997)
			Z93412	Complete genome (strain Hededam)	ssRNA, linear		10,845	Stone *et al.* (1997)
			AF143863	Complete genome (strain 14-58)	ssRNA, linear		10,845	Betts and Stone (2000)
			AF143862	Complete genome (strain 96-43)	ssRNA, linear		10,845	Betts and Stone (2000)
			AB179621	Complete genome strain KRRV9822)	ssRNA, linear		11, 158	Byon *et al.* (2006)
10 Spring viraemia of carp virus (SVCV)	Vesiculovirus	Rhabdoviridae	NC_002803, U18101	Complete genome	ssRNA, linear		11,019	Bjorklund *et al.* (1996)
			EU177782	Complete genome (isolate BJ0505-2)	ssRNA, linear		11,047	Unpublished
			DQ491000	Complete genome (isolate A2)	ssRNA, linear		10,990	Unpublished
			AJ318079	Complete genome (strain ATCC VR-1390)	ssRNA, linear		11,019	Hoffmann *et al.* (2002)
11 Red-spotted grouper nervous necrosis virus (RGNNV)	Betanodavirus	Nodaviridae	NC_008040	Segment RNA 1 (strain SGWak97)	ssRNA, linear	3,105	4,539	Iwamoto *et al.* (2004)
			NC_008041	Segment RNA 2 (strain SGWak97)	ssRNA, linear	1,434		Iwamoto *et al.* (2004)

12 *Epinephelus tauvina* nervous necrosis virus (ETNNV)	Betanodavirus	Nodaviridae	NC_004137	Segment RNA 1 (strain Singapore)	ssRNA, linear	3,103	4,536	Tan *et al.* (2001)
			NC_004136	Segment RNA 2 (strain Singapore)	ssRNA, linear	1,433		Tan *et al.* (2001)
13 Striped jack nervous necrosis virus (SJNNV)	Betanodavirus	Nodaviridae	NC_003448	Segment RNA 1	ssRNA, linear	3,107	4,528	Iwamoto *et al.* (2001)
			NC_003449	Segment RNA 2	ssRNA, linear	1,421		Iwamoto *et al.* (2001)
14 Infectious salmon anaemia virus (ISAV)	Isavirus	Orthomyxoviridae	NC_006505	Segment 1 (isolate 810/9/99)	ssRNA, linear	2,169	12,716	Unpublished
			NC_006503	Segment 2 (isolate CCBB)	ssRNA, linear	2,185		Clouthier *et al.* (2002)
			NC_006502	Segment 3 (isolate CCBB)	ssRNA, linear	2,046		Clouthier *et al.* (2002)
			NC_006501	Segment 4 (isolate CCBB)	ssRNA, linear	1,787		Clouthier *et al.* (2002)
			NC_006500	Segment 5 (isolate CCBB)	ssRNA, linear	1,504		Clouthier *et al.* (2002)
			NC_006499	Segment 6 (isolate CCBB)	ssRNA, linear	1,323		Clouthier *et al.* (2002)
			NC_006498	Segment 7 (isolate CCBB)	ssRNA, linear	966		Clouthier *et al.* (2002)
			NC_006497	Segment 8 (isolate CCBB)	ssRNA, linear	736		Clouthier *et al.* (2002)
Shellfish virus								
1 White spot syndrome virus (white spot bacilliform virus, WSSV)	Whispovirus	Nimaviridae	NC_003225, AF332093	Complete genome (isolate WSSV-CH)	dsDNA, circular		305,107	Yang *et al.* (2001)
			AF440570	Complete genome (isolate WSSV-TW)	dsDNA, circular		307,287	Tsai *et al.* (2004)
			AF369029	Complete genome (isolate WSSV-TH)	dsDNA, circular		292,967	van Hulten *et al.* (2001)
2 Infectious myonecrosis virus (IMNV)	Unclassified	Totiviridae	NC_007915, AY570982	Complete genome (isolate Brazil)	dsRNA, linear		7,560	Poulos *et al.* (2006)
			EF061744	Complete genome (isolate Indonesia)	dsRNA, linear		7,561	Unpublished

(*Continued*)

Table 21.5. *Continued*

Virus name	Genus	Family	Accession no.	Genome shape (strain/ isolation)	Genome type	Length (bp)	Total genome size (bp)	References
Shellfish virus								
3 Taura syndrome virus (TSV)	Cripavirus	Discistroviridae	NC_003005, AF277675	Complete genome	ssRNA, linear		10,205	Mari *et al.* (2002)
			DQ21279	Complete genome (isolate Venezuela)	ssRNA, linear		10,095	Cote *et al.* (2008)
			AY997025	Complete genome (isolate Th04Lv)	ssRNA, linear		10,205	Srisuvan *et al.* (2005)
			DQ104696	Complete genome (isolate ZHZC3TSV)	ssRNA, linear		10,202	Unpublished
4 Infectious hypodermal and haematopoietic necrosis virus (IHHNV)	Brevidensovirus	Parvoviridae	NC_002190, AF218266	Complete genome	ssDNA, linear		4,075	Mari *et al.* (1993)
			EF633688	Complete genome (isolate Fujian)	ssDNA, linear		3,833	Unpublished
5 Hepatopancreatic parvovirus (HPV)	Unclassified	Parvoviridae	NC_007218, DQ002873	Complete genome	ssDNA, linear		6,321	Sukhumsirichart *et al.* (2006)
			NC_011545, FJ410797	Complete genome (isolate India)	ssDNA, linear		6,222	Unpublished
6 *Macrobrachium rosenbergii* nodavirus (MrNV) (host: *Macrobrachium rosenbergii*)	Unclassified	Nodaviridae	NC_005094, AY222839	Segment RNA-1	ssRNA, linear	3,202	4,377	Sri Widada *et al.* (2003)
			NC_005095, AY222840	Segment RNA-2	ssRNA, linear	1,175		Sri Widada *et al.* (2003)

*The structure of iridoviridae genome could be circularly permuted and terminally redundant (Tidona and Darai, 1997).

Table 21.6. Protein sequences coded in the published genome sequences of fish and shellfish infectious viruses.

Virus name	Virus protein	Accession no. (amino acid sequence)	Accession no. (genome)
Fish virus 1 KHV, CyHV-3	ORF1L~ORF8L	YP_001096040~YP_001096047	NC_009127; DQ177346
	ORF9~ORF156	YP_001096048~YP_001096191	
	ORF1R~ORF8R	YP_001096192~YP_001096199	
	Secreted (soluble) TNFR	ORF4L/R,12	
	G protein-coupled receptor	ORF16	
	Deoxynucleotide (deoxyguanosine) kinase	ORF19	
	Small subunit of ribonucleotide reductase	ORF23	
	Predicted membrane glycoprotein	ORF25,26,27,30,40,65, 99,115,116,124, 126,131,136,138,139,146, 148,149	
	Similar to bacterial NAD-dependent epiderase/dehydratase	ORF28	
	Similar to eukaryotic DUF614 proteins	ORF31	
	Similar to a family of Singapore grouper iridovirus proteins	ORF32	
	ATPase subunit of terminase	ORF33	
	Multiple membrane-spanning (multiple transmembrane) protein	ORF29,39,64,81-83,114,153	
	RING-finger protein (SPRY protein; TRIM-like protein)	ORF41,128,144,150	
	Primase	ORF46	
	Similar to protein kinase	ORF48	
	Zinc-binding protein	ORF54	
	Thymidylate kinase	ORF55,140	
	OUT-like cysteine protease domain	ORF62	
	Similar to myosin and related protein	ORF68	
	DNA helicase	ORF71	
	Capsid triplex protein 2	ORF72	
	Capsid protease and scaffolding protein	ORF78	
	DNA polymerase	ORF79	
	Major capsid protein	ORF92	
	Trypsin-like serine protease	ORF94	
	Uracil-DNA glycosylase	ORF98	
	Serine-threonine protein kinase	ORF104	
	Among the least convincing protein-coding regions	ORF105	
	Contains double-stranded nucleic acid-binding domain	ORF112	
	dUTP diphosphatase (dUTPase); deoxyuridine triphosphatase	ORF123	
	Predicted membrane protein	ORF132	
	Interleukin-10	ORF134	
	Ribonucleotide reductase large subunit	ORF141	

(*Continued*)

Table 21.6. *Continued*

Virus name	Virus protein	Accession no. (amino acid sequence)	Accession no. (genome)
Fish virus			
2 CCHV	ORF1~ORF79	NP_041092~NP_041169	NC_001493
	ORF1R~ORF14R	NP_041170~NP_041183	
	Deoxyribonucleoside kinase	ORF5	
	RING-finger protein	ORF9,11,12	
	Membrane glycoprotein	ORF10, 46	
	Protein kinase	ORF14-16,73,74	
	Membrane protein	ORF19, 51	
	Helicase	ORF25	
	Capsid triplex protein 2	ORF27	
	Putative capsid maturational protease	ORF28	
	Major capsid protein	ORF39	
	Subtilisin-like proprotein convertase	ORF47	
	dUTP diphosphatase	ORF49	
	Capsid triplex protein 1	ORF53	
	DNA polymerase catalytic subunit	ORF57	
	Major envelope protein	ORF59	
	Putative terminase ATPase subunit	ORF62	
	Putative primase	ORF63	
	Tegument protein	ORF65	
	Tegument-associated protein	ORF72	
	(Deoxy)nucleoside-phosphate kinase	ORF76	
	Putative Zn-binding protein	ORF78	
3 IPNV	Hypothetical protein	NP_047195	NC_001915 (segment A)
	Polyprotein	NP_047196	
	Viral protein 1 (RNA polymerase)	NP_047197	NC_001916 (segment B)
4 YAV	Protein VP5	YP_899471	NC_004168 (segment A)
	Polyprotein	NP_690805	
	Putative RNA-dependent RNA polymerase	NP_690835	NC_004176 (segment B)
5 LCDV	Proliferating cell nuclear antigen	NP_078615	NC_001824
	DNA methyltransferase	NP_078617	
	DNA-dependent RNA polymerase largest subunit	NP_078624	
	Putative antimutator GTP pyrophosphohydrolase MutT	NP_078631	
	DNA-directed RNA polymerase subunit 2	NP_078633	
	Ribonucleotide reductase small subunit	NP_078636	
	VLTF2-like late transcription factor	NP_078638	
	Papain-like proteinase	NP_078647	
	Hypothetical immediate-early protein	NP_078648	
	Galactose-binding lectin	NP_078654	
	Virion assembly protein, NTPase	NP_078656	
	Collagen-like protein	NP_078660	
	Myristylated membrane protein A	NP_078665	

(*Continued*)

Table 21.6. *Continued*

Virus name	Virus protein	Accession no. (amino acid sequence)	Accession no. (genome)
Fish virus			
	Uncharacterized LCDV1 paralogue family 1	NP_078666, NP_078668, NP_078728	
	Apoptosis regulation Bcl-2 family protein	NP_078671	
	Phosphotransferase	NP_078677, NP_078729	
	Putative NIF/NLI interacting factor	NP_078678	
	Uncharacterized conserved domain linked to protein kinase domain	NP_078689	
	Tristetraprolin-like zinc finger protein C3H	NP_078696	
	Thiol oxidoreductase	NP_078699	
	Ariadne-2 homologue	NP_078700	
	Hypothetical LCDV1 paralogue family 2	NP_078702, NP_078704	
	Early iridovirus protein	NP_078713	
	D5 family NTPase involved in DNA replication	NP_078717	
	SWI/SNF2 family helicase	NP_078720	
	DNA polymerase family B	NP_078724	
	Deoxynucleoside kinase	NP_078725	
	Ribonuclease III	NP_078726	
	Major capsid protein	NP_044812	
	Hydroxysteroid dehydrogenase	NP_078739	
	Membrane (myristylated) protein	NP_078745	
	Putative replication factor and/or DNA binding/packing protein	NP_078747	
	TNF/TNFR- and CUB-domains protein	NP_078749	
	Transcription factor SII homologue	NP_078754	
	Ribonucleotide reductase large subunit	NP_078756	
	Putative filamentous protein	NP_078764	
	Putative XPG/RAD2-type nuclease	NP_078767	
6 SGIV	ORF001~ORF00162 (L/R)	AAS18016~AAS18177	AY521625
	3-beta-hydroxy-delta-5-C27-steroid oxidoreductase	ORF003R	
	Ribonucleoside-diphosphate reductase beta subunit	ORF047L	
	CARD-like protein	ORF048L	
	dUTPase	ORF049L	
	D5 family NTPase	ORF052L	
	NTPase	ORF060R	
	Ribonucleoside-diphosphate reductase alpha subunit	ORF064R	
	Deoxynucleoside kinase	ORF067L	
	Thiol oxidoreductase	ORF070R	
	Major capsid protein	ORF072R	
	DNA-directed RNA polymerase II second largest subunit	ORF073L	
	Tyrosine kinase	ORF078L, 081L	
	RNase III	ORF084L	
	Transcription elongation factor TFIIS	ORF085R	

(*Continued*)

Table 21.6. *Continued*

Virus name	Virus protein	Accession no. (amino acid sequence)	Accession no. (genome)
	Putative immediate-early protein	ORF086R	
	DNA repair protein RAD2	ORF097L	
	Ubiquitin/ribosomal protein	ORF102L	
	DNA-dependent RNA polymerase II largest subunit	ORF104L	
	Putative replication factor	ORF116R	
	DNA polymerase	ORF128R	
	ATPase	ORF134L	
	NTPase/helicase	ORF146L	
	Phosphotransferase	ORF150L	
	Helicase	ORF152R	
7 IHNV	Nucleocapsid protein	NP_042676	NC_001652
	Polymerase-associated protein	NP_042677	
	Matrix protein	NP_042678	
	Glycoprotein	NP_042679	
	Non-virion protein	NP_042680	
	RNA polymerase	NP_042681	
8 VHSV	Nucleoprotein	NP_049545	NC_000855
	Phosphorylated protein	NP_049546	
	Matrix protein	NP_049547	
	Glycoprotein	NP_049548	
	Non-virion protein	NP_049549	
	Large protein	NP_049550	
9 SVCV	Nucleocapsid protein	NP_116744	NC_002803
	Phosphoprotein	NP_116745	
	Matrix protein	NP_116746	
	Glycoprotein	NP_116747	
	Polymerase	NP_116748	
10 RGNNV	Protein A (RNA-dependent RNA polymerase)	YP_611155	NC_008040 (RNA1)
	Protein B	YP_611156	
	Coat protein	YP_611157	NC_008041 (RNA2)
11 ETNNV	RNA-dependent RNA polymerase	NP_689433	NC_004137 (RNA1)
	Hypothetical protein	NP_689434	
	Coat protein	NP_689432	NC_004136 (RNA2)
12 SJNNV	Protein A	NP_599247	NC_003448 (RNA1)
	Protein B	NP_599248	
	Coat protein	NP_599249	NC_003449 (RNA2)
13 ISAV	PB2 polymerase	YP_145807	NC_006505 (segment 1)
	Putative PB1 protein	YP_145804	NC_006503 (segment 2)
	Putative nucleocapsid protein	YP_145803	NC_006502 (segment 3)

Table 21.6.

Virus name	Virus protein	Accession no. (amino acid sequence)	Accession no. (genome)
	Putative PA protein	YP_145802	NC_006501 (segment 4)
	Putative acetylesterase	YP_145801	NC_006500 (segment 5)
	Putative HA protein	YP_145800	NC_006499 (segment 6)
	P4	YP_145798	NC_006498 (segment 7)
	P6	YP_145796	NC_006497 (segment 8)
	P7	YP_145797	
Shellfish virus			
1 WSSV	wsv001 (VP28)	AAL33534	AF332093 (WSSV-CH)
	wsv002~wsv245	AAL33006~AAL33249	
	wsv246	AAL33535	
	wsv247~wsv286	AAL33250~AAL33289	
	wsv287	AAL33536	
	wsv288~wsv436	AAL33290~AAL33438	
	wsv437	AAL33533	
	wsv438~wsv531	AAL33439~AAL33532	
	Putative protein kinase	wsv083	
	Putative CREB-binding protein	wsv100	
	Putative dUTP pyrophosphatase	wsv112	
	Putative ribonucleoside-diphosphate reductase large chain	wsv172	
	Putative ribonucleotide reductase R2 subunit	wsv188	
	Putative deoxyribonuclease I	wsv191	
	Putative nuclear protein	wsv214	
	Putative serine/threonine protein kinase	wsv289	
	Putative transcription initiation factor IID	wsv303	
	Putative thymidylate kinase	wsv395	
	Putative DNA polymerase III catalytic subunit	wsv514	
2 IMNV	Structural protein (coat protein)	YP_529548	NC_007915
	Non-structural protein (gag-pol fusion protein/RNA polymerase)	YP_529549	
3 TSV	Non-structural polyprotein	NP_149057	NC_003005
	Capsid protein precursor	NP_149058	
4 IHHNV	Hypothetical protein (ORF 2)	NP_039237	NC_002190
	Non-structural protein	NP_039238	
	37 kDa coat protein	NP_039239	
5 HPV	Non-structural protein 2	YP_271914	NC_007218
	Non-structural protein 1	YP_271915	
	Structural protein	YP_271916	
6 MrNV	RNA-dependent RNA polymerase	NP_919036	NC_005094 (RNA-1)
	B2 protein	NP_919037	
	Capsid protein	NP_919038	NC_005095 (RNA-2)

predicted 27 membrane proteins (including transmembrane protein or glycoprotein) of KHV are major components of the virion envelope and appear to be relevant for the virus host cells as well as the host immune response (Table 21.6). The vertebrate immune components containing two soluble types of tumour necrosis factor receptor (TNFR) and interleukin-10 are also found in the KHV genome. The KHV envelope protein pORF81 has already been detected by Western blot and immunoelectron microscopy (IEM) using rabbit anti-pORF81 serum (Rosenkranz *et al.*, 2008).

White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV, alternatively named white spot bacilliform virus [WSBV]) is an acute disease pathogen of major economic importance in cultured penaeid shrimp worldwide. The virus is not only present in shrimp but also occurs in other fresh, brackish and seawater crustaceans, including crabs and crayfish (Lo *et al.*, 1996). WSSV is a large, double-stranded circular DNA virus assigned to the genus *Whispovirus* (family *Nimaviridae*), which is not related to any known viruses. The whole genome sequence of WSSV has been determined from three strains isolated from China (WSSV-CH, Yang *et al.*, 2001), Taiwan (WSSV-TW, Tsai *et al.*, 2004) and Thailand (WSSV-TH, van Hulten *et al.*, 2001; Marks *et al.*, 2004). The genome sizes of these strains are 292,967 bp (WSSV-TH), 305,107 bp (WSSV-CH) and 307,287 bp (WSSV-TW) (shown in Table 21.5). The three genomes share an overall nucleotide identity of 99.32%. Compared with WSSV-CN, major differences include a 12-kb deletion in WSSV-TH that is located at 275,235–287,285 (the nucleotide coordinates are from WSSV-CN), a variable region at 267,203–268,046, and an insertion of a 1.3 kb transposase sequence in WSSV-TW at 204,978–204,979 (Marks *et al.*, 2004, 2005). The WSSV genome has nine homologous repeat regions, each of which contains numerous imperfect palindromic repeats (250 bp in size) (van Hulten *et al.*, 2001; Yang *et al.*, 2001; Marks *et al.*, 2004). The number of non-overlapping ORFs (≥ 60 amino acids) ranges from 181 (in the WSSV-CN genome) to 184 (in the WSSV-TH genome). Some genes encode proteins with identifiable function. These proteins include DNA replicating enzymes (DNA polymerase, ribonucleotide reductase subunits, dUTPase, thymidylate synthase, thymidine-thymidylate kinase) and protein-modifying proteins (protein kinase) (Table 21.6). Proteomic studies on purified virions have led to the identification of over 50 structural proteins (Huang *et al.*, 2002; Tsai *et al.*, 2004; Zhang *et al.*, 2004; Xie and Yang, 2006; Li *et al.*, 2007). Together with other techniques, such as Western blot analysis and IEM, these structural proteins could be classified into envelope, tegument and nucleocapsid proteins (Tsai *et al.*, 2006; Xie *et al.*, 2006). The structural proteins include a very large gene, *vp664*, that encodes a major nucleocapsid protein of about 664 kDa (Leu *et al.*, 2005). Shotgun proteomic analysis of WSSV infected epithelium identified 11 novel proteins that were presumed to be non-structural (Wu *et al.*, 2007). Functional studies have meanwhile discovered several WSSV genes, including latency-related genes (Khadijah *et al.*, 2003), immediately early genes (Liu *et al.*, 2005), ubiquitination-related genes (Wang *et al.*, 2005; He *et al.*, 2006) and anti-apoptosis genes (Wang *et al.*, 2004; He *et al.*, 2006). However, most ORFs of WSSV are still unassigned.

Acknowledgements

Special thanks to Professor Chu-Fang Lo (Institute of Zoology, Taiwan National University) for giving us advice and comments on the white spot syndrome virus.

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Glossary

Abrasion: superficial injury to skin or mucous membrane from scraping or rubbing.

Abscess: a focal pocket of necrotic cell debris from cells and tissue disintegration caused by pyrogenic organisms; it may be surrounded by phagocytic and/or fibrocytic haemocytes.

Acetylcholinesterase: an enzyme that degrades the neurotransmitter acetylcholine found at neuromuscular junctions and cholinergic synapses.

Acid-fast bacteria: bacteria that possess a physical property of decolorization resistance to acids during staining procedures.

Acquired immunity: a host defensive response developed following recovery from a prior exposure to a specific infectious agent (or group of agents): it is specific and is mediated by antibody and/or T-cells.

Acute infection: disease that develops rapidly to a crisis.

Adductor muscle: the muscle that is responsible for closing the two shells of a bivalve mollusc; it is also known as the 'meat' of a scallop.

Adhesion: (Crustacea): a pathological condition where destruction of cuticle by chitinolytic bacteria or fungi causes binding of subcuticular tissues to the cuticle. This condition disrupts growth and may impede moulting.

Adhesion: (vertebrate): connective tissue proliferation within and around an organ causing it to attach to the peritoneal or pericardial walls.

Adjuvant: medicament that, when administered with or before an antigen, heightens the immune response.

Aerobactin: a bacterial siderophore found in *E. coli*; virulence factor enabling *E. coli* to sequester iron in an iron-poor environment.

Aerogenic: gas-producing bacteria.

Aerolysin: a channel-forming protein produced by *Aeromonas* spp. that uses glycosylphosphatidylinositol-anchored proteins on target cells. Aerolysin forms heptamers that insert into and produce channels in the cell membrane to cause cell death.

Aetiologic agent: an organism that initiates or causes disease in an animal.

Aetiological diagnosis: any procedure for recognition of a disease by identification of the infectious or causal agent.

Aetiology: the study of the cause of disease, including the factors that enhance transmission and infectivity of the aetiologic agent.

Agglutinin: specific factors present in sera that agglutinate or clump organisms or particulate protein matter.

Agglutinin titre: the concentration of antibody at the greatest dilution capable of causing specific clumping or agglutination of cells or particles.

Albumin: a class of proteins present in many animal and plant tissues.

Allelic exchange: exchange between two forms (sequences) of a particular gene.

Amplified fragment length polymorphism (AFLP): a highly sensitive PCR-based method used to detect DNA polymorphisms.

Anadromous: fish that migrate from the sea to fresh water to spawn.

Anaemia: a condition characterized by deficiency of haemoglobin, packed cell volume and/or erythrocytes.

Anaerogenic: produces a minute amount of or no gas.

Anamnestic response: the ability of an animal to mount an immune response in recognition of a previous exposure to a specific antigen.

Anneal: binding molecules by linkage affinity; frequently applied to DNA and RNA coupling for polymerase chain reaction.

Anorexia: the loss or deficiency of appetite for food.

Antennal gland: a gland (syn. kidney gland, excretory organ or green gland) with excretory pores that open at the base of the antennae in Crustacea.

Antheridium: a haploid structure or organ producing and containing male gametes.

Antibiotic resistance: the capability of a microbe to evade destruction by an antibiotic.

Antibody (Ab): a protein capable of cross-reacting with an antigen. In vertebrates, Ab is produced by lymphoidal cells, especially plasma cells, in response to antigenic stimulation. The exact mechanism of antibody production in shellfish is not known, but is haemolymph mediated.

Antigen: a substance (e.g. protein, lipopolysaccharide) or cell that, under favourable circumstances, elicits an immune response in an organism. An antigen may consist of several epitopes (surface molecules) to which antibody can bind (*see* Monoclonal antibody *and* Polyclonal antibodies).

Antigenic determinant: the site on an antigen molecule that determines the specificity of the evoked antibody.

Antisense RNA: single-stranded RNA that is complementary to a messenger RNA (mRNA) strand transcribed within a cell.

Antiserum (pl. antisera): serum containing antibodies induced against one, or more, specific antigens.

Apical plate: a dorso-central plate (in Echinoderms) surrounded by five plates in a radial pattern. One of the five plates incorporates the excretory pore (madreporite) and all are associated with the genital pores.

Aplanospore: a non-motile sporangiospore.

Apoptosis: cell death following a sequence of events which leads to elimination of the cell without the release of inflammatory compounds into the surrounding area.

Apparent prevalence: the proportion of test-positive fish in a target population.

Ascites: abnormal accumulation of serous fluid in the abdominal cavity.

Aseptate: hyphae that do not have internal cross walls.

Atrophy: a decrease in the amount of tissue, or size of an organ, after normal growth has been achieved.

Attention: a process by which organisms are rendered less virulent by exposure to an unfavourable environment.

Attenuated: rendered less virulent, the alteration of a pathogenic microorganism to decrease virulence in its native host.

Attributable rate: the absolute difference between the rates of disease in the exposed and the non-exposed groups.

Authentic strain: the reliable or authorship strain.
Autointerference: impaired reproduction of a virus in cell culture caused by competition for available replication factors.
Autolysis (-lytic): rupture of cell membranes due to autolytic enzymes (contained in the lysosomes of eukaryotes or in the cell walls of prokaryotes). Autolysis may be a normal function of cell replacement.
Auxotrophic mutant: a mutant strain of a microorganism that proliferates only when the medium is supplemented with specific substance not required by the wild-type or parent organisms; a nutritional-requirement mutant.
Avirulent: an infectious agent that causes negligible or no disease in a host organism.
Axenic culture: a culture containing cells of a single species (e.g. bacterial culture) or cell type (tissue culture).

Bacillus Calmette-Guérin (BCG): a tuberculosis vaccine prepared from the attenuated live *Mycobacterium bovis*.
Back passage: a series of *in vivo* passages of a microorganism in a host to demonstrate its pathogenic property.
Bacteraemia: the (abnormal) presence of bacteria in blood.
Bacterial artificial chromosome (BAC): a DNA construct, based on a functional fertility plasmid (or F-plasmid), used for transforming and cloning in bacteria, usually *E. coli*.
Bactericidin: an antibody or enzyme capable of killing bacteria.
Bacterin: a bacterial culture that has been killed or inactivated using chemical or other means and is used as a vaccine; simplest form of bacterial vaccine.
Bacteriocidal (bactericidal): an organism or a molecular compound capable of killing bacteria.
Bacteriophage: a virus that infects bacteria.
Bar-bodies: organelles that form early in sporangium differentiation prior to septum formation.
Basement membrane: the extracellular supporting layer of mucopolysaccharides and proteins underlying the epithelium (syn. basal membrane and basal lamina).
Basophilic: cellular structures that have an affinity for basic staining solutions under specific pH conditions.
Beta (β)-haemolytic: a haemolysin that causes a clear zone around a bacterial colony grown on blood agar.
Bias: in statistical terms, the difference between the value of a population parameter and the expected value of the estimates of that parameter.
Bicapsid: a virus with a double protein coat surrounding the nucleic acid or nucleoprotein core; usually with icosahedral symmetry. The bicapsid layer may be surrounded by an envelope.
Bikont: a eukaryotic cell with two flagella.
Bioassay: a procedure that uses susceptible organisms to detect toxic substances or pathogens.
Biofilm: a microbial film formed on surfaces and comprising of multiple layers of bacterial cells. In monospecific biofilms, properties of the cells at the bacteria–liquid interface may differ substantially from those at the bacteria–surface interface.
Biosecurity: a best-practice protocol for protecting animals from contamination by disease-causing agents, and for preventing the spread of diseases.
BLAST (basic local alignment search tool): algorithm for comparing primary biological sequence information, such as the amino acid sequences of different proteins or the nucleotides of DNA sequences.
BOX PCR: repetitive sequence PCR using a BOX A1R primer for genetic fingerprinting of microorganisms.
Brush border: absorptive surface of gut cells consisting of finger-like projections.

Budding: fusion of a viral capsid with a host membrane to incorporate the lipid component of the host membrane into the viral envelope; the protein component of the envelope remains viral.

Cachexia: a state of constitutional disorder, malnutrition and general ill health.
Capsid: a protein coat surrounding the viral nucleic acid or nucleoprotein core. Capsids usually have icosahedral symmetry and may be surrounded by an envelope.
Capsomere: the protein units that together form the capsid of a virus.
Capsule: a compact layer of polysaccharide exterior to the cell wall in some bacteria.
Carapace: exoskeletal covering of the thorax and anterior trunk (in Crustacea) arising from a posterior fold of the head cuticle.
Cardiomyopathy: a disease or disorder of the heart muscle, especially of unknown or obscure cause.
Carrier: an animal with no clinical disease but infected persistently with a pathogen; pathogens from carrier fish may be shed in faeces, reproductive products or urine.
Caseation: necrosis in which tissue is changed into a spongiform mass.
Caspase: aspartate-specific cysteine protease (as inactive or low activity proenzymes) in cells that are sequentially activated to promote apoptosis.
Caspase cascade: a series of caspases that must be activated in stepwise sequence to promote apoptosis.
Catadromous: fish that migrate from inland waters to the sea to spawn.
Catalase: an enzyme responsible for detoxifying H_2O_2 produced by aerobic metabolism; it is absent from most obligate anaerobic bacteria.
Catarrhal enteritis: an acute inflammation of the intestine characterized by increased secretion of mucus and faecal casts.
Catenulate: in chains, end to end.
Cecropins: peptide antibiotics derived from the haemolymph of some insects following bacterial challenge.
Cell line: the *in vitro* cultivation of animal cells; it includes a cloned, tumorous or transformed cell source.
Cellular immunity: specific immune reactions mediated by T lymphocytes, via their elaboration of biologically potent cytokines, and/or the induction of phagocytic cells.
Cellulitis: diffuse inflammation of the soft or connective tissue due to infection, in which a thin, watery exudate spreads through the cleavage planes of interstitial and tissue spaces.
Chaperonin: protein complexes that assist the folding of nascent, non-native polypeptides into their native, functional state.
Chelating agent: an agent that strongly binds divalent cations (e.g. Zn^{2+}, Ca^{2+} and Mg^{2+}).
Chemiluminescence: a chemical reaction that results in the emission of light.
Chemotherapeutant: any chemical that is used to treat an infection or non-infectious disorder.
Chemotherapy: the use of a specific chemical agent to arrest the progress of, or eradicate, disease in an animal without causing irreversible injury to healthy tissues.
Chitin: a linear polysaccharide found in the exoskeletons of arthropods, the cell walls of most fungi, certain algae and the cyst walls of ciliates.
Chitinolytic (chitinoclastic): chitin-degrading organisms that have chitinase enzyme capable of breaking down the chitin component of arthropod exoskeletons.
Chlamydospore: an irregularly shaped or spherical hyphal swelling that is enclosed by a thickened cell wall (asexual spore formation).
Chondroitin: a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (*N*-acetylgalactosamine and glucuronic acid).

Chorion: the outermost membrane or layer covering the egg; also known as the eggshell in fish.

Chromalveolata: a eukaryote supergroup, comprised of organisms descending from a bikont that performed secondary endosymbiosis with a red alga.

Chromatin: a nucleoprotein complex containing genomic DNA and RNA in the nucleus of most eukaryotic cells.

Chromatophores: motile, pigment-containing epidermal cells of cephalopod molluscs, responsible for skin-colour change.

Chromoshift: colour change of bacterial colony growth in the presence of alkali; usually from yellow to orange.

Chronic infection: constant or long lasting without causing an acute disease condition.

Ciliostatic exotoxin: a toxin secreted by a bacterium that inhibits the function of ciliated cells.

Clinical signs: signs of a sick animal that can be observed.

Cloned DNA probe: radioactively or chemically labelled copies of a defined DNA segment; serves for the location and identification of an identical DNA sequence by hybridization.

Coelomocytes: haemocytes of echinoderms.

Collagen: the principal proteinacous substance in connective fibres.

Complement: a group of proteins belonging to the defence system, present in blood serum; it enhances viral neutralization.

Conchiolin: a nitrogenous albuminoid substance that is usually dark brown and forms the organic base of molluscan shells.

Concurrent infection: infections comprising more than one infectious species; some infectious agents may have a synergistic effect, while others may have a repressive effect on other infectious agents.

Conidium (-a): a sexually derived, non-motile spore formed from a specialized conidium-forming cell (e.g. sporangiospore).

Conjugation: the transfer of genetic information from a donor cell to a recipient cell by cell-to-cell contact.

Conjugative plasmid: a self-transmissible plasmid that encodes all the functions needed for its own intercellular transmission by conjugation.

Contagious: a disease normally transmissible only by direct contact between infected and uninfected organisms.

Convalescent serum: the serum taken from an animal that is recovering from a disease.

Corpuscles of Stannius: small glands located on the kidneys of teleost fish; removal of these glands causes an elevation of plasma calcium levels.

Corticosteroid: a hormone produced by the adrenal cortex that influences the inflammatory defence response.

Cosmid: a type of hybrid plasmid that contains *cos* sequences that can be used to build genomic libraries.

Cosmid library: genomic library made with cloning vectors containing the *cos* sites of the lambda bacteriophage.

Cowdry bodies: nuclear inclusion bodies attributed to viral infections. Type A nuclear inclusions are amorphous or particulate and condensed in spherical masses which do not stain like nucleoli. Type B inclusions are localized in certain areas of the nucleus and the nucleoplasm may not be altered significantly.

Cristae: internal vesicular or tubular structures of mitochondria usually supporting ribosomes.

CRP protein (catabolite repression protein): protein that controls gene expression in response to changes in metabolic breakdown products known as catabolites.

Cuticle: a proteinaceous structure (in Crustacea) consisting of an outer layer (epicuticle), an underlying exocuticle (pigmented layer), endocuticle (calcified layer) and membranous uncalcified layer. Chitin occurs in all but the epicuticle.

Cyst: (a) a non-motile, resistant, dormant stage of a free-living or a parasitic organism, or (b) a host-response that walls off a tissue irritant or infection.

Cytokines: regulatory proteins (such as the interleukins and lymphokines) that are released by cells of the immune system and they act as intercellular mediators in the generation of an immune response.

Cytolysin: a toxin that lyses cells.

Cytopathic effect (CPE): *in vitro* cell damage typically caused by viral infections. The type of CPE produced in cell cultures is often characteristic of a particular virus group, e.g. cells lysing leaving cellular debris, or cells fusing to produce multinucleated cells (syncytia); the end result is often cell death.

Cytotoxic necrotizing factor: toxins that are produced by certain pathogenic strains of *Escherichia coli*.

Dedifferentiation: reversion of the specified cell function of a mature cell to a non-specified function similar to that of a germ cell. Characteristic of neoplastic cell changes (*see* Neoplasia).

Defective interfering particles (DIP): viral particles whose nucleic acids lack part of the viral genome; these particles often interfere with the replication of the normal virus.

Demersion: exposure to air following immersion in water.

Deoxyribovirus (DNA-virus): virus with a deoxyribonucleic acid (*see* DNA) genome (*see* Ribovirus).

Detection level: the lowest concentration of an antigen that can be detected by a given diagnostic test.

Determinant: a factor that plays a causal role in outcomes such as disease, death and production loss.

Deuteromycete: fungi belonging to the non-phylogenetic group Deuteromycotina, originally created for fungi with no known sexual stage of development (also known as Fungi Imperfecti). Asexual production is via conidia; some deuteromycetes are now known to undergo sexual reproduction, as well as form conidia, but are still included in the Deuteromycotina.

Diads (syn. diplococci): covalent binding of two coccoid bacteria to form pairs.

Diapedesis: the movement of haemocytes across intact epithelia to void metabolic products, toxins or infectious organisms from the body.

Diclinous: having the oogonium and its antheridium on different hyphae.

Dimorphic: having two body types.

Diplanetic: a condition in which there are two motile stages with a resting stage in between.

Direct fluorescent antibody test/technique: an immunodiagnostic technique that uses antibody labelled with a fluorescent dye to indicate antibody binding to a specific antigen.

Direct prophylaxis: prophylaxis based on disinfection of the culture facilities.

Discharge tube: a tube produced by protistan or fungal agents through which motile zoospores are released from sporangia.

Disease outbreak: localized group of individuals infected with a pathogen; on a larger scale it may be referred to as an epizootic.

Disinfection: a process by which a material or solution is rendered free of infectious agents.

DNA (ssDNA, dsDNA): nucleic acid comprised of deoxyribonucleotides containing adenine, guanine, cytosine and thymine. Single-strand DNA (ssDNA) occurs in some viruses (usually as a closed circle); however, in eukaryotes and many viruses, the

DNA is double-stranded (dsDNA) with complementary base pairing between opposite strands.

DNA hybridization: a technique used to determine relatedness between two or more separate strands of DNA from different species of organisms.

DNA probes: segments of DNA from an organism used to identify homologous segments of DNA from another organism; these probes can be used for rapid and specific identification of infectious organisms (*see* RNA probes).

DNA vaccine: a technique to protect an organism from disease by injecting it with genetically engineered DNA, usually of a pathogen.

Ecchymose: the escape of blood from a blood vessel into surrounding tissue: visible as a red to purple spot somewhat larger than in petechial bleeding.

Ecchymosis: the passage of blood from ruptured blood vessels into subcutaneous tissue; it is marked by a purple discoloration of the skin.

Ecdysal gland (Crustacea): *see* Y-organ.

Ectoparasite: a parasite that has adapted to live on the outside surface of its host.

Ectoplasmic net: a branching, anastomizing network of membrane-bound extrusions, produced by labyrinthulids and thraustochytrids, which is believed to release lytic enzymes.

Elementary body: a rigid-walled infectious stage of development of Chlamydiales that develops into a thin-walled reticulate (initial) body within infected cells. These divide to form intermediate bodies, which in turn condense to form infective elementary bodies.

ELISA (enzyme-linked immunosorbent assay): an immunoassay used to detect antigen (antigen-capture ELISA) or antibody (antibody-capture ELISA).

Emaciation: excessive leanness, or wasting of body tissue.

Emphysematous: a pathological accumulation of air or gas in tissues or organs.

Empyema: accumulation of pus in a cavity of the body.

Encapsulation: the covering of a parasite by the host with materials mostly, if not entirely, of host origin.

Encephalitis: inflammation of the brain.

Encephalon: the term denoting brain.

Encephalopathy: any degenerative disease of the brain.

Encystment: the covering of a parasite with materials of parasite origin.

Endemic: an infection that is well established in a geographical location or within a population, and thus has a stable prevalence or incidence rate.

Endocardium: the endothelium forming the inner lining of the heart.

Endocrine: denotes the release of glandular products into the bloodstream.

Endocuticle (Crustacea): the calcified layer of the cuticular exoskeleton, lying between the exocuticle and a proteinaceous structure consisting of an outer layer (epicuticle), an underlying basement membrane.

Endoplasmic reticulum: a network of fine tubules in the cytoplasm, which form the structural framework and circulation pathway of a cell.

Endosymbiotic: the association between two organisms (one living within the other) where both organisms derive benefit from the association or live together without obvious adverse effect.

Endothelium: the single-celled inner layer of the circulatory system (heart, blood vessels, lymph vessels, capillaries).

Enophthalmia: abnormally sunken eyes.

Enteritis: inflammation of the intestine.

Enterobacterial repetitive intergenic consensus (ERIC)-PCR: also referred to as intergenic repeat units (IRUs); these are imperfect extragenic palindromic sequences which are 127 nucleotides long, form a stem-loop structure and can be oriented.

Enterohaemolysin (Ehly1): a plasmid-encoded *Escherichia coli* toxin that readily causes haemolysis.

Enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF): diarrhoeagenic *Escherichia coli* that produce a characteristic histopathology known as attaching and effacing (A/E) on intestinal cells and that do not produce Shiga, Shiga-like or verocytotoxins.

Enterotoxin: a protein toxin released by a microorganism into the intestine that is frequently cytotoxic and kills cells by altering the permeability of the epithelial cells of the intestinal wall.

Envelope: a lipoprotein membrane composed of host lipids and viral proteins (principally glycoproteins). In some viruses, the envelope may be ornamented by spines. Non-enveloped viruses are composed solely of the capsid and nucleoprotein core.

Eosinophilic: cellular structures that have an affinity for acidic staining solutions.

Epibionts: organisms (bacteria, fungi, algae, etc.) that live on the surfaces (*see* Fouling) of other living organisms.

Epicardium: the membranous external layer of the heart; also known as the visceral or internal layer of the sac around the heart.

Epicuticle: the non-chitinized outer layer of cuticle covering the exocuticle and endocuticle.

Epidemiology: the study and description of the frequency, distribution and determinants of health and disease in human populations.

Epipodite (Crustacea): the cuticular extension of the base (protopodite) of the walking legs (pereiopods).

Epitope: the small portion of the antigen molecule that fits specifically within the cleft of the antibody binding site.

Epizootic: major outbreak of a disease that either affects a large number of animals or is spread over a large area.

Epizootic ulcerative syndrome (EUS): a seasonal epizootic condition in freshwater and estuarine warmwater fish of complex infectious aetiology characterized by invasive *Aphanomyces* infection and necrotizing ulcerative lesions, typically leading to a granulomatous response.

Epizootiology: the study of the factors influencing infection by a pathogenic agent (syn. epidemiology – used for the disease processes of human pathogens).

Erythemic: inflammation, redness of the skin.

Erythrodermatitis: a pathological condition of the skin characterized by intense, widespread reddening of the dermis that is preceded or associated with exfoliation.

Escape mutant: a cell-culture viral mutant that has lost the capacity to be neutralized by either a polyclonal or monoclonal antiserum; likely due to a nucleotide coding change at the virus-neutralizing epitope.

Ethylenediamine di (o-hydroxyphenylacetic acid): an organic compound $[C_2H_4(NH_2)_2]$ widely used as a building block in chemical synthesis.

Exocrine: denotes the excretory release of glandular products.

Exocuticle (Crustacea): the pigmented layer of the exoskeleton that is between the epicuticle and endocuticle.

Exoenzyme: an extracellular enzyme released by a cell or microorganism.

Exophthalmia: abnormal protrusion of the eyeball from the orbit, especially caused by disease. Also known as 'popeye'.

Exoskeleton: the chitin and calcified outer covering of crustaceans (and other arthropods) which protects the soft tissues.

Extracellular: produced by the cell (e.g. a bacterial pathogen), eventually occurring outside the cell, for example, proteases.

Extramatricial hyphae: vegetative tubular structures growing out from the body of the infected organism. Where the hyphae form an interconnecting network, the structure is known as the aerial mycelium.

Exudate: a protein-rich fluid released during the inflammatory processes or from tissues subjected to circulatory disturbance.
Eyed eggs: the stage of a fish embryo when two dark spots (eyes) appear on the egg; these eggs are robust and can be transported.
Eye-stalk ablation (Crustacea): removal of the eye-stalk to prevent production of moult-inhibiting hormone (*see* Y-organ).

Fastidious: bacteria with specific nutritional needs that require specified growth media.
Fatty acid methyl ester (FAME): a unique profile inherent in every microorganism; used as a tool in microbial source tracking.
F-cells (Fibrillenzellen): hepatopancreas cells of crustaceans that are of unknown function, but may be precursors of B-cells (Blasenzellen), which produce digestive enzymes (*see also* R-cells).
Fermentation: anaerobic metabolism usually of organic substrates that results in no net oxidation of the substrate (e.g. lactic acid fermentation, proprionic acid fermentation).
Feulgen (-positive, -negative): specific stain for DNA in tissue sections. Feulgen-positive areas react with Schiff 's reagent to give a red colour.
Fibroblasts: connective tissue cells responsible for synthesis of collagen.
Fibrosis: proliferation of connective tissue containing a high proportion of fibroblasts.
Fimbrial protein: a family of bacterial proteins that are involved in regulation of length and mediation of adhesion of fimbriae. Fimbriae (also called pili) are polar filaments radiating from the surface of the bacterium to a length of 0.5–1.5 micrometres that enable bacteria to colonize the epithelium of specific host organs.
Fixed phagocytes: phagocytic cells embedded in the walls of the hepatopancreas (digestive gland) arterial sinuses in invertebrates.
Flagella: whip-like appendages on the surface of some bacterial species usually associated with motility and attachment. Flagella may be distributed around the cell (peritrichous), or at one or both ends of rod-shaped cells (polar).
Flexuous: having turns or windings, having opposite alternate curvatures.
Flow cytometry: a method for optical recognition of suspended cells that are passed through a capillary vessel, with discrimination potential based on size and/or specific labelling.
Fluorescent antibody technique (FAT): the use of fluorescently conjugated antibodies, or an antibody detection system incorporating fluorescent molecules to visualize antigen-bearing cells or molecules under microscopic examination.
Fluorescent *in situ* hybridization (FISH): a technique in which a deoxyribonucleic acid or ribonucleic acid probe is labelled with a fluorescent dye (that can be visualized under a fluorescent microscope) and then hybridized with target genetic material.
Focal lesion: a small lesion in any tissue, limited to a focus.
Fouling: any organism that adheres to the hard surfaces of submerged materials or organisms.

Gametangium: an organ or cell in which gametes are produced; found in many multicellular protists, algae or fungi.
Gametocytic: a substance or organism that kills gametocytes or germinal cells.
Ganglioneuritis: inflammation of nerves adjacent to the brain.
G+C content: the percentage of nitrogenous bases on a DNA molecule that are either guanine or cytosine; it is variable with different organisms.
Gemmae (chlamydospore): thick-walled, intercalary or terminal asexual spore made by the rounding up of a cell or cells.
Genetic drift: changes in the genotype of an isolated population of a species due to persistent mutations that neither enhance nor reduce survival.

Genetic selection: natural or artificially selected genotypes that enhance species survival (natural selection) or species productivity (commercial or artificial selection). Natural selection for disease resistance may be accelerated by overselection of disease survivors. Survivors are challenged with purified pathogens and the survivors of this second challenge are selected as disease-resistant broodstock.

Genetic variation: differences in alleles of genes that occur within and between populations.

Genogroup, genotype: related viruses within a genus; may be further subdivided into genetic clusters.

Genome: the total genetic information of an individual.

Genome segment: a part of the genomic DNA or RNA.

Genomospecies: phylogenetic definition of a species, generally would include strains with 70% or greater DNA–DNA relatedness at optimal conditions and with 5°C or less ΔDT_m.

Germling: a germinated spore.

Gill lamellae: consist of primary lamellae that are attached to the gill arch and secondary lamellae that run perpendicular to the axis of the primary gill filaments.

Glial cells (Crustacea): cells lying between the neurilemma and the giant neurosecretory cells of the nerve ganglia.

Glucan: polymer of glucose molecules, including linkages between constituents.

Glycocalyx: coating on a bacterial cell surface rich in carbohydrate moieties.

Glycoprotein: any protein with one or more covalently linked oligosaccharide chains.

Gold standard: a diagnostic test with close to 100% sensitivity and specificity.

Grading: farming practice of periodically sorting fish stock into size classes to prevent cannibalism and/or to facilitate feed management and later harvesting.

Granulocytes: (a) in vertebrates – granular leucocytes (eosinophils, basophils and neutrophils); (b) in invertebrates – haemocytes with or without eosinophilic cytoplasmic granules (granular or agranular, respectively) and with a higher nucleoplasm and cytoplasm volume ratio than hyalinocytes (*see* Hyalinocytes). Granular granulocytes are often called phagocytes; however, agranular granulocytes and hyalinocytes also have phagocytic capabilities.

Granuloma (syn. granulocytoma): an aggregation of granulocytes (tumour) that may or may not involve epithelial or connective tissue elements.

Granulomatous lesion: a lesion that is formed due to the elicitation of an intense cellular immune response, usually to a foreign antigen or pathogen. It is characterized by substantial infiltration of phagocytic cells that cause damage to infected and surrounding normal tissue.

Granulosis virus: baculoviruses belonging to the subgroup (B) characterized by a single nucleocapsid within an envelope. Granulosis viruses form intranuclear ellipsoid or rounded occlusion bodies (granules or capsules) containing one or two virions.

Haem: a red organic pigment containing ferrous iron present in haemoglobin.

Haemagglutination: the agglutination of red blood cells.

Haematocrit: the proportion (%) of the blood volume occupied by red blood cells; also often called packed cell volume (PCV).

Haematoma: swelling caused by blood accumulating in the interstitial tissues following capillary rupture or vascular leakage. Accumulated blood usually demonstrates some degree of clotting.

Haematopoietic tissue: a sheet of tissue composed of small lobules (in decapod Crustacea) surrounded by fibrous connective tissue which lies along the dorso-lateral surfaces of the posterior portion of the cardiac stomach (Brachyura) or surrounding the lateral arterial vessels, secondary maxillipeds and epigastric tissues (Penaeidae and Nephropidae).

Haematopoietic tissues: tissues involved in formation of cellular components of the blood. In teleost fish, this includes the anterior (head) kidney (pronephros), spleen and thymus.

Haematoporphyrin: a porphyrin that does not contain iron and is formed by acidic decomposition of haem or haemoglobin.

Haemoconcentrated blood: increased concentration of red blood cells, or decrease in plasma relative to a constant red blood cell count.

Haemocytopenia: a reduction in the number of cells in the circulatory system. Usually associated with a reduction in blood-clotting capability.

Haemocytosis (syn. haemolysis): lysis or destruction of haemocytes.

Haemolymph: the haemocytes and serum composing the circulatory fluid in invertebrates.

Haemolysis: the disruption of red blood cells and release of their haemoglobin.

Haemorrhage: (a) internal or external bleeding in invertebrates caused by rupture of blood vessels. Capillary haemorrhaging within a tissue may cause tissue displacement (*see* Haematoma); (b) uncontrolled loss of haemocytes in invertebrates due to tissue trauma or epithelial rupture (*see* Diapedesis).

Halophile: an organism whose growth is enhanced or is dependent on supplementation with salt (NaCl) for growth.

Helical symmetry: the symmetry of a rod-shaped virus where the capsomere is aligned in the shape of a helix.

Hepatopancreas (Crustacea): a digestive organ or gland composed of digestive ducts and tubules.

Heritability: the proportion of phenotypic variation in a population that is attributable to genetic variation among individuals.

Heterokont: having flagella of different lengths.

Heterologous virus: a virus derived from a species with a different genetic constitution.

Heterothallic: condition of sexual reproduction in which 'conjugation' is possible only through the interaction of different thalli.

Histolysis: the breakdown of tissue by disintegration of the plasma membranes.

Homothallic: both male and female sexual reproductive structures appearing on a single mycelium.

Horizontal transmission: infection of the individuals by non-vertical routes (i.e. via water, feed, or fish or vectors).

Humoral: associated with body fluids.

Humoral immunity: specific immune reactions mediated by B lymphocytes via their elaboration of antibodies.

Hyalinocytes (syn. lymphocytes): invertebrate haemocytes characterized by a low nucleoplasm to cytoplasm volume ratio, a polymorphic nucleus with one or more distinct nucleoli, numerous mitochondria, endoplasmic reticulum and few cytoplasmic granules.

Hydrocephalus: the abnormal accumulation of fluid in ventricles of the brain: in fish it includes progressive bulging of head in the median region behind eyes, local thinning of the skull, atrophy of the brain and nervous disorders.

Hydrophobic domain: a region of a polypeptide chain that contains a concentration of apolar residues along the sequence. These domains are normally found in the unexposed parts of the protein or in membrane-bound segments of the polypeptide chain.

Hyperaemia: the localized congestion of tissue with blood, manifested by abnormally intense red coloration.

Hyperimmune serum: the blood serum with high levels of antibodies obtained from an experimental animal treated by a programme of intense immunostimulation.

Hyperosmotic infiltration: a method of mass vaccination for fish; it consists of two consecutive short baths. Hypertonic solution of sodium chloride enhances the entrance of antigen into the fish by the lateral line and possibly through skin and gills.

Hyperplasia: an abnormal increase in size of a tissue or organ due to an increase in number of new cells (*see* Neoplasia).
Hypertrophy: an increase in size of a tissue or organ due to an increase in size of individual cells.
Hyphae: tubular cells of filamentous fungi. The tubules may be divided by cross-walls (*see* Septum) into multicellular hyphae, and may be branched. Interconnecting hyphae are called mycelia.
Hypophysation: the injection of broodfish with a preparation from whole fish hypophysis containing gonadotropic hormones to induce ovulation in females and spermiation in males.
Hypoxia: oxygen deficiency in tissues or organs.

Icosahedral: a symmetrical polyhedron consisting of 20 equilateral triangles.
Immune complex: an aggregate of antigens and their specific antibodies that, if not eliminated, can induce extensive damage by the activation of complement and induction of phagocytic digestion.
Immunization (syn. vaccination): the protection of an organism against disease by exposure to pathogen antigens so that the defence system recognizes and produces a rapid and effective response against the same antigens on subsequent exposures.
Immunoassay: a biochemical test that detects or measures the concentration of specific antigen or antibody in samples by means of an antigen–antibody interaction.
Immunoblot: an antibody-based method for the detection of antigens that have been fixed to a solid support such as nitrocellulose.
Immunocompetent: a state in an organism describing its ability to mount an immune response.
Immunodepression: the decrease in response of the immune system to antigens because of an established infection (same or different agent) or exposure to an immunosupressant chemical.
Immunogenicity: the ability of a molecule (antigen) to induce an immune response.
Immunoglobulin (Ig): a family of proteins made up of light and heavy molecular chains linked together by disulfide bonds; usually produced in response to antigenic stimulation.
Immunohistochemistry: a method for the detection of antigens in tissues by using specific antibodies.
Immunoprophylaxis/indirect prophylaxis: the induction of an immune response and protection by vaccination against an infectious organism.
Immunoreactive: the ability of a molecule or antigen to react with components of the immune system such as serum or lymphocytes.
Immunostimulation: specific or non-specific enhancement of the defence responses, e.g. with vaccination.
Immunotoxicity: the property of a substance to cause toxic damage to the immune system.
Inactivated vaccine: a vaccine containing an antigenically active infectious agent whose infectivity has been destroyed by chemical or physical means.
Incidence rate: a fraction used to quantify the occurrence of new cases of a disease or an infection (morbidity rate) or death (mortality rate).
Inclusion body: non-specific discrete bodies found within the cytoplasm or nucleus of a cell. Usually composed of virions (*see* Cowdry bodies *and* Polyhedral inclusion/occlusion body). Intracellular bacterial inclusions are usually referred to as microcolonies.
Incubation period: the lag time between establishment of an infection and the onset of clinical disease.
Induction: a mechanism stimulating (*see* Promoter) genetic transcription of a specific enzyme or protein required for growth or survival (syn. operon). The induction process

is controlled by a repressor that stops production when there is an excess of the product.

Infectious: capable of causing an infection in an animal.

Inflammation: (a) the initial response in invertebrates to tissue injury; this is characterized by the release of amines which cause vasodilation, infiltration of blood cells and proteins, and redness; (b) in invertebrates, it is haemocyte infiltration in response to tissue damage or a foreign body. The infiltration may be focal, diffuse or systemic.

Innate immunity: host defence response to a pathogen that does not require prior exposure to the pathogen.

***In situ* hybridization**: a hybridization technique that detects and localizes the presence of a specific genetic target sequence within tissue samples when a labelled deoxyribonucleic acid or ribonucleic acid probe binds with it by complementary base pairing.

Interferon gamma: a dimerized soluble cytokine that is the only member of the type II class of interferons; originally called macrophage-activating factor.

Interferons: a family of antiviral agents secreted *in vivo* and *in vitro* by virus-infected cells; they protect neighbouring cells and thus the organism from superinfection by virus.

Internal transcribed spacer (**ITS**): refers to a piece of non-functional RNA situated between structural ribosomal RNA.

Interstitial tissue (cells): tissue or cells found in between epithelial bound organ systems; sometimes referred to as Leydig tissue (molluscs) or connective tissue.

Intimin: a virulence factor in *E. coli* strains (O127:H6; O157:H7).

Intracellular components (ICC): substances produced and contained within the cell membrane.

Intramatricial hyphae: hyphal tubes penetrating the tissues of an infected host.

Intrapallial: the space between the mantle, gills and other soft tissues in bivalves. The space between the mantle and the inner shell surface is the extrapallial space.

Iodophor: an iodine carrying chemical substance and a solubilizing agent that releases free iodine when in solution.

Isogenic: a characterization based on essentially identical genes.

Kappa: a quotient, which varies between zero and one, that measures the amount of agreement between two diagnostic tests or two diagnosticians.

Karyorrhexis: the rupture of the cell nucleus.

Kinetosome (blepharoplast): the basal body or granule from which arise the longitudinal fibres constituting the axoneme of a flagellum.

Kinetosome-derived vesicles (K2 bodies): encystment vesicles, formerly known as bar-bodies.

Latent infection: a persistent infection where, after an acute primary phase, the pathogen apparently disappears since infectious virus cannot be isolated. However, viral antigens are produced. At a later time, reactivation results in the production of infectious virus.

Lectins: sugar-binding proteins that are highly specific for their sugar moieties and typically play a role in biological recognition phenomena involving cells and proteins.

Leptomeningeal: related to the two innermost layers of tissues that cover the brain and spinal cord (leptomeninges).

Lesions: abnormal changes in tissues or body functions.

Lethal dose 50 (LD50): the dose that is required to kill 50% of the population.

Leucopenia: an abnormal decrease in the number of leucocytes.

Lipopolysaccharide: complex lipid structure containing unusual sugars and fatty acids found in many Gram-negative bacteria, and constituting the chemical structure of the outer layer.

Liposome: a spherical vesicle composed of a lipid bilayer membrane.

Loop-mediated isothermal amplification (LAMP): a single tube technique for the amplification of DNA that can be combined with a reverse transcription step to allow the detection of RNA.
Lordosis: an exaggerated forward, convex curve of the lumbar spine.
Luminescent bacteria: marine bacteria which contain the autoinducible, fluorescent enzyme, luciferase, under certain conditions, e.g. *Vibrio harveyi* and *V. splendidus*.
Luminol-dependent chemiluminescence (LDCL): a test to measure the oxidative capacity of haemocyte defences. Light is emitted when the compound is exposed to unstable oxygen radicals (oxidative by-products).
Lymph: colourless fluid derived from blood by permeation through walls of capillaries.
Lymphatic spaces: ducts in which lymph circulates and which communicate with veins.
Lymphoid: pertaining to lymph or tissues of the lymphoid system.
Lyophilization: freeze-drying.
Lysin: an antibody that causes cell lysis (e.g. haemolysin) or a bacterial toxin that lyses (ruptures) cell membranes.
Lysosome: a cytoplasmic organelle that produces lytic enzymes to break down cells or microorganisms that have been phagocytosed by the cell.
Lytic activity: disintegration of cells.

Macroconidia: large (multinuclear) asexual spores (conidia) produced singly or in clusters along the sides or at the tips of specialized hyphae (conidiophores). Small uninuclear conidia are called microconidia.
Macroglobulins: large molecular weight proteins found in the plasma, e.g. $\alpha 2$ macroglobulin.
Macrophage: a large amoeboid blood cell, responsible for phagocytosis, inflammation, antibody and cytotoxin production in vertebrates.
Major histocompatibility complex (MHC): a large genomic region or gene family found in most vertebrates that plays an important role in the immune system, autoimmunity and reproductive success.
Mandibular organ (Crustacea): a large glandular organ close to the ventral epidermis between the mandibles. It is believed the function is related to the moulting cycle, although the organ does not produce a known moult-inducing hormone.
Marker assisted selection (MAS): the selection of breeding animals based on the presence or absence of a genomic marker.
Melanin: dark brown-black polymer pigment that has enzyme-inhibiting properties. It forms part of the primary defence mechanism against cuticle and epidermal damage in many crustaceans.
Melanization: the process by which melanin is deposited in or around damaged tissues.
Melanomacrophage centre: group of melanomacrophages normally found in spleen, kidney and liver of fish.
Melanophores (syn. melanocytes): dermal cells containing melanin.
Membrane cisternae: the space between the membranes of the flattened sacs of the endoplasmic reticulum, Golgi bodies and nuclear envelope of eukaryote cells.
Meninges: membranes that surround the central nervous system.
Meningitis: inflammation of the membranes that surround the brain or spinal cord.
Meningoencephalitis: inflammation of the brain and meninges.
Mesencephalon: the term denoting midbrain.
Mesenteries: any of several folds of the peritoneum that connect the intestine to the abdominal wall.
Mesophilic: refers to an organism that prefers growth at moderate temperatures.
Messenger RNA (mRNA): an RNA molecule that specifies the amino acid sequence during protein synthesis.
Metalloprotease: a proteolytic enzyme whose catalytic mechanism involves a metal.

Metastasis: the transportation of particles (including microorganisms and cells) from a primary focus of disease to another, distant body part by conveyance through blood or lymph vessels.
Microbiota: microbial communities associated with a particular environment or ecosystem, including environments and ecosystems internal to the host, such as the gastrointestinal tract.
Microcolonies (syn. inclusion bodies): membrane-bound population of reticulate bodies of *Chlamydia* bacteria or non-membrane bound rickettsial colonies.
Microcyst: a small cyst or spore.
Molecular phylogenetics (also known as molecular systematics): the use of the structure of molecules to gain information on an organism's evolutionary relationships. The result of a molecular phylogenetic analysis is expressed in a phylogenetic tree.
Monocistronic: it refers to mRNA carrying the information for the synthesis of only a single protein translation product.
Monoclonal antibody: an antibody derived from a single B-cell clone that has been immortalized by fusion with a myeloma fusion partner. The resultant cell is a hybridoma.
Moribund: dying or close to death.
Moulting (syn. ecdysis) (Crustacea): the shedding of the exoskeleton to facilitate growth of internal soft tissues.
Mucins: mucopolysaccharide secretions with bacterial binding properties (as well as digestive system functions).
Mucoid: relating to or resembling mucus; 'a mucoid substance'.
Multinucleated giant cell: a cell with many nuclei present in chronic inflammation lesions, especially in granulomatous inflammation.
Multinucleosis (Crustacea): the presence of a number of multinucleate (syn. polynuclear) cells in the haemolymph.
Multiple aetiology: a disease associated with more than one infectious agent.
Multiplex PCR: the amplification of multiple target sequences in one and the same PCR cycling protocol by addition of more than one set of oligonucleotide primers.
Multiplex polymerase chain reaction (PCR): the simultaneous amplification of multiple regions of DNA templates by adding more than one primer pair to the amplification reaction mixture.
Multiplicity of infection (MOI): refers to the number of infectious virions per cell used to initiate infection.
Mutator-type transposase: autonomous mobile genetic elements such as transposon or insertion sequences (IS) encode an enzyme, transposase, that is required for excising and inserting the mobile element.
Mx protein: a host protein that inhibits viral replication; it is induced directly by interferon via upregulation of *Mx* gene expression.
Mycelial colonies: colonial growth of Gram-positive actinomycete bacteria with branched mycelia that may fragment into rods or coccoid forms.
Mycelium: the vegetative part of a fungus or oomycete, consisting of a mass of branching, thread-like hyphae.
Mycosis: a disease resulting from infection by a fungus.
Myodegeneration: breakdown of muscle fibres.
Mysis larvae (Crustacea): pelagic stage of larval development between protozoea (zoea) and postlarva.

Nacre: the inner calcareous layer of molluscan shells. May have an iridescent crystal matrix (mother-of-pearl).
Natural killer cells: a type of cytotoxic lymphocyte that is a major component of the innate immune system.

Nauplius (Crustacea): the earliest stage of crustacean larval development characterized by three pairs of appendages, uniramous first antennae, biramous second antennae and mandibles.

Necrobiotic bodies: cellular debris found in the blood, characteristic of infection with infectious haematopoietic necrosis virus.

Necrosis: localized death and degeneration of cells and tissues in a living organism. The process is irreversible and involves nuclear breakdown (*see* Pyknosis) followed by cell death.

Neoplasia (neoplastic): uncontrolled cell proliferation that lacks structural or functional coordination. A neoplasia may develop into a discrete tumour (benign) or continue to grow (malignant, e.g. carcinoma).

Nested-PCR: a technique that involves two sets of primers used in two successive runs of polymerase chain reaction; the second set is intended to amplify a secondary target within the first-run product.

Non-ribosomal peptide synthetase: an enzyme that catalyses the addition of amino acids independently of the translational process.

Normocytic aplastic anaemia: a decrease in the number of mature red blood cells accompanied by increased numbers of immature red blood cells with abnormal morphology.

Nuclear polyhedrosis viruses (NPV): baculoviruses (Type A) that produce intranuclear polyhedral protein matrices (*see* Polyhedral inclusion/occlusion body).

Nuclease: an enzyme acting upon nucleic acids.

Nucleocapsid: a protein–nucleic acid complex that may form the core, capsid and/or helical nucleoprotein of the virion.

Nucleotides: units that form DNA and RNA, composed of a purine or pyrimidine base linked to a ribose or a deoxyribose sugar with one or more phosphate groups.

Number at risk: the number of units that at any given point in, or period of, time are biologically capable of, or susceptible to, experiencing the event of interest.

Occlusion: blockage of the vascular sinuses in invertebrates by haemocytes; (perivascular) infiltration of haemocytes, several cells deep into the tissues surrounding vascular sinuses; (luminal) filling or blocking of gonadoducts, renal ducts, digestive tubules or ducts by haemocytes or other cell debris.

Ochrophyta: a phylum that encompasses the photosynthetic chromistan (chlorophyll a,c containing) algae, such as diatoms, phaeophytes and xanthophytes.

Odds ratio: the ratio between the odds of disease in the exposed and in the non-exposed groups or between the odds of exposure in the diseased and in the non-diseased groups.

Oedema: swelling of a tissue or of an organ caused by excessive accumulation of serous fluid in extracellular spaces due to increased permeability of capillaries.

OIE (the World Organization for Animal Health): an intergovernmental organization responsible for improving animal health worldwide that is recognized as a reference organization by the World Trade Organization (WTO). The OIE provides the *Aquatic Animal Health Code* and the *Manual of Diagnostic Tests for Aquatic Animals*.

Oligonucleotide: a short polynucleotide chain (DNA or RNA).

Oogonium: an immature ovum; it is a female gametogonium.

Oomycete (or water moulds): a group of filamentous, unicellular Heterokonts, physically resembling fungi.

Oomyceticide: a chemical compound that is used to kill or inhibit oomycetes.

Oospore: a sexual spore produced by the union of two morphologically different gametangia (oogonium and antheridium).

Open reading frame (ORF): a sequence of bases that potentially encode a protein. It is located between the initiation codon and the termination codon of a gene.

Operon: a functioning unit of key nucleotide sequences of DNA including an operator and one or more structural genes that is controlled as a unit to produce messenger RNA, in the process of transcription by an RNA polymerase.
Opportunistic pathogen: an organism capable of causing disease only when a host's resistance is lowered by other factors (another disease, adverse growing conditions, drugs, etc.).
Opsonin: any molecule (antibody) that binds to foreign particles or organisms and increases their susceptibility to phagocytosis.
Optic tectum: the centre for sight that occupies most of the forebrain in fish.
Ori: site of initiation of DNA replication.
Osteitis: inflammation of the bone.
Outer membrane proteins (OMPs): a wide variety of biological molecules, primarily proteins and lipids, found on the cell membrane of cells that are involved in a vast array of cellular processes such as cell adhesion, ion channel conductance and cell signalling.
Oxidase test: a test used to detect cytochrome c and the associated oxidative enzyme, oxidase, in bacteria. Used to differentiate oxidative (aerobic) from fermentative (anaerobic) bacteria.
Ozone treatment: a method to disinfect water; a free radical of oxygen, which readily gives up an atom of oxygen to produce a strong oxidizing agent that is toxic to microbes.

Papilloma: a branching benign tumour derived from epithelium.
Papule: small fleshy projection.
Paracrystalline arrays (bodies): geometrically arrayed aggregations of particles (usually viruses).
Paraspherical: nearly spherical.
Paratenic host: a host capable of harbouring a live infective agent without supporting its proliferation.
Parenteral: denoting any route (such as intravenous, subcutaneous, intramuscular or mucosal) other than the alimentary canal.
Parenteral challenge: infection challenge administered via a route other than the digestive tract.
Parthenogenetically: the apomictic development of haploid cells.
PAS reaction: the periodic acid-Schiff (PAS) reaction renders cellulose, glycogen and starch carbohydrates red and is used to detect glycogen, mucin, basement membranes and certain fungi in tissue sections.
Patent infection: infection period when clinical signs and/or the infectious organism can be detected (*see* Prepatent infection).
Pathogenicity: the capacity of an organism to produce disease.
Pathognomonic: clinical signs that are indicative or characteristic of a specific disease.
Paucity: smallness of quantity.
Pentasaccharide: a carbohydrate that, on hydrolysis, yields five molecules of monosaccharides.
Peptides: short polymers formed from the linking of α-amino acids in a defined order.
Peptidoglycan: a bacterial cell wall polymer that consists of an N-acetylglucosamine and N-acetylmuramine acid sugar backbone, which are cross-linked by short peptide chains.
Pereiopods: thoracic appendages of crustaceans ('walking legs') (*see* Pleopods *and* Uropods).
Pericardium: the membrane sac that encloses the heart in vertebrate animals.
Periostitis: inflammation of the periosteum.
Periostracum: outer covering of calcareous layers of molluscan shell that may contain quinine-tanned protein.

Peritoneum: the serous membrane that lines the walls of the abdominal cavity and folds inward to enclose the viscera.

Peritonitis: the inflammation of the peritoneum, i.e. of the serous membrane lining the interior of the peritoneal cavity and its contained viscera.

Perivascular tissue: the thin layer of tissue surrounding a blood vessel.

Perivasculitis: inflammation of a perivascular sheath and surrounding tissue.

Persistent infection: a condition in which the virus is not cleared but remains in specific cells of infected individuals. It may involve stages of both silent and productive infection without rapidly killing or even producing excessive damage to host cells.

Petechiae: small red spots on an organ or body surface, caused by minute haemorrhage.

Petechial haemorrhage: small or minute reddish spots on the skin or in the serous or mucous membranes caused by bleeding.

Phenotype: observable physical or biochemical characteristics of an organism.

Phenotypic phenotype: the outward expression of a gene(s) as an observable characteristic or activity (e.g. enzymatic).

Phylogenetic analysis: the analysis of the interrelationships between genes of organisms based on nucleic acid sequences. The product is a phylogenetic tree or cladogram.

Phylogeography: a field of study that analyses the geographical distribution of genealogical lineages. It uses DNA sequence variation between individuals across a species range to reconstruct gene genealogies.

Plaque: more or less clear (and usually circular) areas in a bacterial lawn or confluent cell monolayer that result from cell fusion or the killing or lysis of cells by several cycles of viral replication.

Plaque-forming unit (pfu): a measure of the viral titre as the number of plaques produced per unit volume.

Plasmid vector: a plasmid is a linear or circular molecule of DNA that can replicate independently from the chromsonal DNA of an organism. If a portion of DNA is added to that of a plasmid, the sequence can be added to a cell where it can replicate and alter the host genome.

Pleiotropic: effect of a single gene on multiple phenotypic traits.

Pleomorphic: an organism demonstrating more than one body form within a life cycle.

Pleomorphism: denotes a wide range in shape and size of individuals in a population.

Pleopods: abdominal appendages of crustaceans ('swimmerets') (*see* Pereiopods *and* Uropods).

Poikilotherms: vertebrates whose body temperature fluctuates with the ambient temperature.

Polyadenylated RNA: messenger RNA (mRNA) that has a polyadenylate sequence bound to the 3' end of the molecule. This is common in most eukaryote mRNA and is present in some riboviruses.

Polycistronic: messenger RNA containing more than one coding region.

Polyclonal antibody (PAb): an antiserum prepared from an organism exposed to an antigen. The PAb contains several different antibodies, each specific to a different epitope of the same antigen (*see* Monoclonal antibody (MAb)).

Polyclonal antisera: sera derived from an immunized animal, containing a wide variety of antibodies (from many B-cell clones) to the inducing antigen(s).

Polyhedral inclusion/occlusion body (PIB, POB): protein-based crystalline matrix made up of polyhedrin [baculorivus group A – nuclear polyhedrosis viruses (NPV)] or granulin [baculovirus group B – granulosis viruses (GV)]. Baculovirus group C do not form occlusion bodies.

Polymerase chain reaction (PCR): the selective amplification of DNA by repeated cycles of heat denaturation of the DNA, annealing of two oligonucleotide primers that flank the sequence to be amplified, and the extension of the annealed primers with a heat-stable DNA polymerase.

Polymorphic: (a) the ability of biomolecules, such as enzymes, to exist in more than one molecular form; (b) the ability of nuclei of certain cells (e.g. haemocytes) to change shape; (c) the ability of microorganisms to change shape (e.g. in different host species or tissues).

Polyplanetism: repeated cycles of encystment and emergence following release of secondary zoospores and the failure to locate a suitable host or substrate.

Population attributable rate: the attributable rate multiplied by the prevalence of the risk (exposure) factor in the target population.

Population parameter: the true aggregated value of some characteristic in a target population at a point in or period of time, e.g. the true average or total of some variable or the true rate of disease.

Postlarva(e): the pre-adult stage where the morphology is similar to that of the adult (shrimps and lobsters, not crabs). Follows nauplius, protozoea and mysis stages of development.

Power: in experiments, field trials or analytic observational studies, the probability of not failing to reject the null hypothesis when it is false.

Precipitin: an antibody that forms a precipitate when bound to its reacting antigen.

Predisposing factors: causes that weaken the host and render it liable to infection and/or disease.

Preening: crustacean behaviour for cleaning surface tissues or eggs exposed to fouling (*see* Epibionts *and* Fouling).

Prepatent infection: the period between infection and the manifestation of clinical or detectable signs of disease.

Prevalence: the percentage of animals in a population that are infected at any one time by a specific organism.

Primary cyst: an encysted primary zoospore.

Primary zoospore: zoospore that is released from a sporangium.

Probiotics: dietary supplements containing potentially beneficial bacteria or yeasts.

Pro-inflammatory: capable of promoting inflammation.

Promoter: a nucleotide sequence, within a DNA strand, which initiates transcription of RNA.

Propagule: the part of an organism that may be disseminated to reproduce the organism.

Prophylactic (-axis): action or chemotherapeutant administered to healthy animals to prevent infection (*see* Treatment).

Proportional rate: a fraction in which the denominator includes only part of the population at risk.

Protease: a protein-hydrolysing enzyme.

Proteogenomics: the use of proteomic data for gene annotation.

Pustule: a sub-epidermal swelling containing necrotic cell debris as a result of inflammation in response to a focal infection. The pustule may also contain living or dead infectious organisms.

Pyknosis: a condition in which the nucleus of a cell stains more deeply than normal, and is thought to be a precursor of necrosis.

Pyknotic: the shrinking of a cell nucleus resulting in the formation of a small, dense staining body of nucleoplasm. The process is irreversible and results in cell death (*see* Necrosis).

Quantitative RT-PCR (qRT-PCR): a molecular technique that uses the general principle of RT-PCR; its key feature is that the amplified cDNA (complementary DNA) is quantified as it accumulates in the reaction in real time after each amplification cycle.

Quantitative trait locus (QTL): a genome locus being associated with a heritable quantitative trait.

Quorum sensing: molecular mechanism by which bacteria sense cell density.

Random amplified polymorphic DNA (RAPD): a DNA typing technique using markers that are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence, and which are able to differentiate between genetically distinct individuals.

R-cells (Restenzellen) (Crustacea): the absorptive and storage cells of the hepatopancreas.

Reactive oxygen species (ROS): ions or very small molecules that include oxygen ions, free radicals and peroxides, both inorganic and organic.

Real-time PCR (quantitative real-time PCR): a technique used to amplify and quantify simultaneously a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample.

Reassortant virus: hybrid virions (new strains) containing segments from different viruses produced in cells infected with different strains of the virus.

Recombinant virus: a virus formed by recombining genetic material.

Refractory: able to resist disease.

Relative risk: the ratio between the risk rate in an exposed group and the risk rate in a non-exposed, or reference, group.

Reniform: kidney-like in form.

Residual bodies: a lysosome that has completed digestion of a phagocytosed particle.

Residual compounds: compounds formed between residual oxidants and cations in the aquatic environment (e.g. chloramines are formed using free chlorine ions in seawater). These compounds are usually toxic to shellfish.

Residual oxidants: anions (usually halogens) that bind to organic compounds (oxidize) and are lethal to microorganisms (e.g. chlorine, iodophors, hydrogen peroxide and ozone).

Resistance: the capacity of an organism to control the pathogenic effects of an infection. Resistance does not necessarily negate infection ('refraction') and varying degrees of tolerance to the infection may be manifest.

Restriction enzyme analysis (REA): a method that exploits the specificity of restriction enzymes to digest either the entire genome or specific genes of an organism into unique and discernible patterns.

Restriction enzymes: enzymes that cut or digest DNA at very specific nucleotide sequences.

Restriction fragment length polymorphism (RFLP): differing lengths of DNA fragments produced by a restriction endonuclease that cleaves at a polymorphic locus. Such variations are created by mutations that alter recognition sites for these enzymes.

Reticuloendothelial cells: cells of common ancestry and fulfilling many vital functions, e.g. defence against infection, antibody, blood cell and bile pigment formation. Main sites of these cells are spleen, liver and lymphoid tissue.

Retinopathy: a pathological disorder of the retina.

Reverse transcriptase-based polymerase chain reaction amplification (RT-PLR): the first strand synthesis from an RNA template is made with reverse transcription. These cDNA transcripts are then amplified by standard polymerase chain reaction procedures.

Reverse transcriptase (or transcription)-polymerase chain reaction (RT-PCR): a highly sensitive molecular technique for the detection and quantitation of all types of RNA, but typically mRNA.

Rhizoid: colony development from a central core in actinomycete bacteria. The term is also applied to fungi to describe root-like hyphal development.

Ribosomes: intracytoplasmic granules that are rich in RNA and function in protein synthesis.

Ribotyping: fingerprinting of genomic DNA restriction fragments that contain all or part of the genes coding for the 16S and 23S RNA.

Ribovirus (RNA-virus): a virus with a ribonucleic acid (*see* RNA) genome (*see* Deoxyribovirus).

Risk incidence rate: the number of cases divided by the number of potential cases at the start of the study period minus half of the withdrawals, excluding cases, during the study period.

RNA (ssRNA, dsRNA): ribonucleic acid consisting of ribonucleotides made up of the bases adenine, guanine, cytosine and uracil. A single strand of RNA (ssRNA) occurs in some viruses (usually as a closed circle). In eukaryotes and many viruses, DNA is double stranded (dsDNA) via base pairing between attractant (complementary) bases of opposite strands.

RNA fingerprinting: a technique for comparing the nucleotide sequences of fragments of RNA from different sources.

RNA probes: segments of ribosomal RNA from an organism that are labelled to identify homologous segments of DNA from another organism. These probes can be used for rapid and specific identification of infectious organisms.

RNAse protection assay: a technique to identify individual RNA molecules in a heterogeneous RNA sample.

Rostrum: extension of the mid-dorsal cephalothoracic carapace anteriorly in front of the eyes in Crustacea.

rRNA: the RNA component of the ribonucleoprotein organelle responsible for protein synthesis within a cell (prokaryote and eukaryote).

Sampling unit: the level (fish, holding unit, farm site or body of water) that is selected by formal random processes in a probability sample.

Sanguineous: pertaining to blood.

Saprobiont: an organism that obtains its nutrients from dead organic matter.

Scoliosis: a lateral curvature of the spine.

Secondary cyst: an encysted secondary zoospore.

Secondary lamellae (gill): off each filament (primary lamellae) are numerous secondary lamellae, which greatly increase the surface area exposure to water and oxygen; small blood capillaries flow through the secondary lamellae.

Secondary zoospore: a zoospore that has been released from a primary cyst.

Sensitivity (of screening tests): the probability that a (randomly selected) infected animal will yield a positive test result.

Sepsis: the presence of pathogenic microorganisms or their toxins in the blood or other tissues.

Septicaemia: presence of bacteria in the circulation system following an infection.

Septum (-a): cross-wall(s) found within fungal hyphae.

Sequence similarity: refers to sequences that are similar in contrast to sequence homology, which refers to evolutionarily related sequences stemming from a common ancestor.

Serine protease: a protease in which one of the amino acids at the active site is serine.

Serodiagnostic technique: a presumptive or confirmatory test that uses antiserum.

Serological relatedness: antigenic similarities of two organisms.

Serosal: a thin membrane or cell layer that is moistened with fluid resembling blood serum.

Serovariant: a variant group of microorganisms that can be distinguished on the basis of their antigenic properties. The differences are reflected, in the case of viruses, by a difference in neutralization kinetics rather than the absence of neutralization as in serotype.

Shiga toxin: a family of related toxins with two major groups, Stx1 and Stx2. The most common sources for Shiga toxin are the bacteria *S. dysenteriae* and the Shigatoxigenic group of *Escherichia coli* (STEC), which includes serotype O157:H7 and other enterohaemorrhagic *E. coli*.

Shucking: the procedure by which a bivalve mollusc is removed from its shell.
Siderophore: a small molecule that complexes with ferric iron and supplies it to the cell by aiding in its transport inside the cytosol.
S-layer (also the A-layer in *Aeromonas salmonicida*): part of the cell envelope consisting of a monomolecular layer composed of identical proteins or glycoproteins.
Smolts: fish (Salmonid) that have gone through the necessary physiological changes to live in salt water and are ready for or actively migrating from fresh water to the sea.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility.
Somatic: pertaining to the body.
Specificity (of screening tests): the probability that a (randomly selected) non-infected animal will yield a negative test result.
Specific pathogen free (SPF): a group of animals shown to be free from certain microorganisms through a specific diagnostic test programme.
Sporangiophore: a specialized hypha bearing one or more sporangia.
Sporangium: the cell, or part of a cell, which subsequently develops into an endospore (intracellularly formed spore).
Spore: the infective stage of an organism that is usually protected from the environment by one or more protective membranes.
Streptavidin: a protein produced by *Streptomyces avidini* that is analogous to the glycoprotein avidin. It has a high binding affinity for biotin and is typically conjugated to alkaline phosphatase or horseradish peroxidase for use in assays such as ELISA, immunoblots and immunohistochemistry.
Stress: an adverse stimulus that results in disruption of physiological balance in an affected organism.
Subcentric: having the central cytoplasm surrounded by one layer of fat droplets on one side and by two or three layers on the other.
Subclinical: infection where no clinical signs of disease are apparent.
Subgenomic RNA: essentially smaller sections of the original transcribed template strand.
Superinfection: a renewed infection following a preceding infection.
Superoxide dismutase: an enzyme that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide.
Survey: a descriptive study designed to describe one or more characteristics in a population, by calculating point and interval estimates from randomly selected samples.
Susceptible: an organism that has no immunity or resistance against infection by another organism.
Syncytium: a protoplasmic mass with many nuclei formed by coalescence of contiguous cells (by cell fusion); formation of syncytia characterizes the cytopathic effects of some viruses.
Syndrome (syn. pathognomic/pathognomonic): an assembly of clinical signs which, when manifest together, are indicative of a distinct disease or abnormality.
Synergistic: increased pathology produced as a result of two or more concurrent infections by different agents, when compared with their individual effects.
Systemic infection: the presence of an infectious agent in an organism.

TaqMan PCR: real-time PCR assay using TaqMan probes developed by Applied Biosystems to increase the specificity.
Telson: terminal segment of the abdomen in Crustacea that overlies the uropods.
Test: calcareous covering enclosing the soft tissues of echinoderms.
Thallus: a branching form of vegetative growth that shows no differentiation into branch or stem-like structures.

Tight junction: the seal between adjacent epithelial cells occurring in a narrow band just beneath their apical surface.

Tissue culture infectious dose ($TCID_{50}$): the highest dilution causing cytopathicity in 50% of tissue cultures inoculated with virus ($TCID_{50}$/ml: a virus titre of an inoculum; $TCID_{50}$/g: a tissue virus titre.)

Toroid: applies to an electron dense oval surrounding, but not touching, a nucleoprotein core that is rod-shaped (bifacies virus type A).

Toxin: a poisonous substance secreted by certain organisms and capable of causing toxicosis when introduced into the body tissue, but also capable of inducing a counteragent or an antitoxin.

Toxoid: a toxin that has lost toxicity but retains the capacity to stimulate the immune response.

Transcriptional fusion: gene construct that investigates transcription activity of a gene of interest with a promoterless reporter gene.

Transfection: the transformation of the genetic code of a cell using isolated viral DNA as a vector.

Transferrin: a glycoprotein in the blood that binds iron.

Transmission: passage of an infectious agent from one host to another.

Transposable element: a genetic element that has the ability to move (transpose) from one site to another on a chromosome.

Transposition: the movement of a piece of DNA on a chromosome, usually through the function of a transposable element.

Transposition mutagenesis: insertion of a transposon into a gene; this inactivates the host gene, leading to a mutant phenotype, and also confers the phenotype associated with the transposon gene.

Transposon: a type of transposable element which, in addition to genes involved in transposition, carries other genes; often conferring selectable phenotypes such as antibiotic resistance.

Treatment: action taken to eradicate an infection (*see* Prophylaxis).

True incidence rate: the number of cases divided by the average number of potential cases times the duration of the study period.

Tumour: abnormal growth as a result of uncontrolled cell division (*see* Neoplasia) of a localized group of cells.

Ubiquitous: occurring everywhere, ability to exist is many places.

Ulcer: the excavation of the surface of an organ or tissue, involving sloughing of necrotic inflammatory tissue.

Unilateral (lesions)/bilateral: refers to one- or two-sidedness of an injury, wound or pathological change caused by a pathogen.

Urea nitrogen: the quantity of urea in serum expressed as its nitrogen.

Uropods: the terminal appendages underlying the telson of crustaceans that form the 'tail fan' (*see* Pereiopods *and* Pleopods).

Vaccine: an antigen preparation from whole or extracted parts of cells, usually an infectious organism, which is used to enhance the specific immune response of a host susceptible to infections by the organism.

Vacuolated: containing spaces or cavities within the cytoplasm of a cell.

Vacuolation: the formation of vacuoles.

Vector: an organism (generally a parasite) that carries or transmits a pathogenic agent from an infected fish to another fish; blood-sucking fish parasites transmit viruses passively (without viral replication).

Veliger: ciliated planktonic larva of a mollusc.

Velum (velar): ciliated surface of veliger larvae.
Vertical transmission: infection of offspring by direct incorporation of the pathogen into the gametes of the parents.
Viable but non-culturable (VBNC): a state in which Gram-negative bacteria no longer grow on conventional media, but remain intact and retain viability; a living microorganism that cannot be cultured.
Viraemia: the substantial presence of a virus in circulating blood.
Viral plaque: the focal cytopathic effect caused by a single infectious virus particle in a cell culture overlaid with semisolid medium (*see also* Plaque-forming unit).
Virion: an intact virus particle.
Virogenesis: production of virions.
Virogenic stroma(e) (syn. viroplasm): viral inclusion body(ies) that is(are) the site of viral replication or assembly.
Virulence: the ability of a microorganism to cause disease.
Virus: a non-cellular microorganism that is an obligate parasite, consists of protein and RNA or DNA, and which can replicate and metabolize solely within a host cell.
Virus neutralization test: the technique for identification of virus isolates by the use of antibodies to a known virus.
Virus reactivation: the mechanism by which latent viruses are induced to replicate and then released as infectious viruses.

Well boats: boats that are used for transportation of live fish.
Western blot (syn. immunoblot): a procedure in which electrophoresed proteins are blotted on to membranes and visualized by staining with antibodies coupled to enzymes or some form of chromogenic or photogenic system.

Y-organ: (syn. ecdysal gland): gland responsible for production of the moulting hormone, ecdysone. Production of the moulting hormone is controlled by a moult-inhibiting hormone synthesized in the eye-stalk.

Ziehl-Neelsen: a special staining technique using basic fuchsin to dye the acid-fast bacteria.
Zoea larvae: stage of larval development following metamorphosis from the nauplius stage in Crustacea. Usually characterized by the emergence of four pairs of thoracic appendages from the carapace.
Zoonotic: a disease transmissible from animals to man under natural conditions.
Zoo-sanitary: relating to the prevention of disease transmission between animals.
Zoosporangium: a sporangium that produces zoospores.
Zoospore: a motile asexual spore utilizing a flagellum for locomotion.
Zoosporogenesis: the formation of zoospores in a sporangium.

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