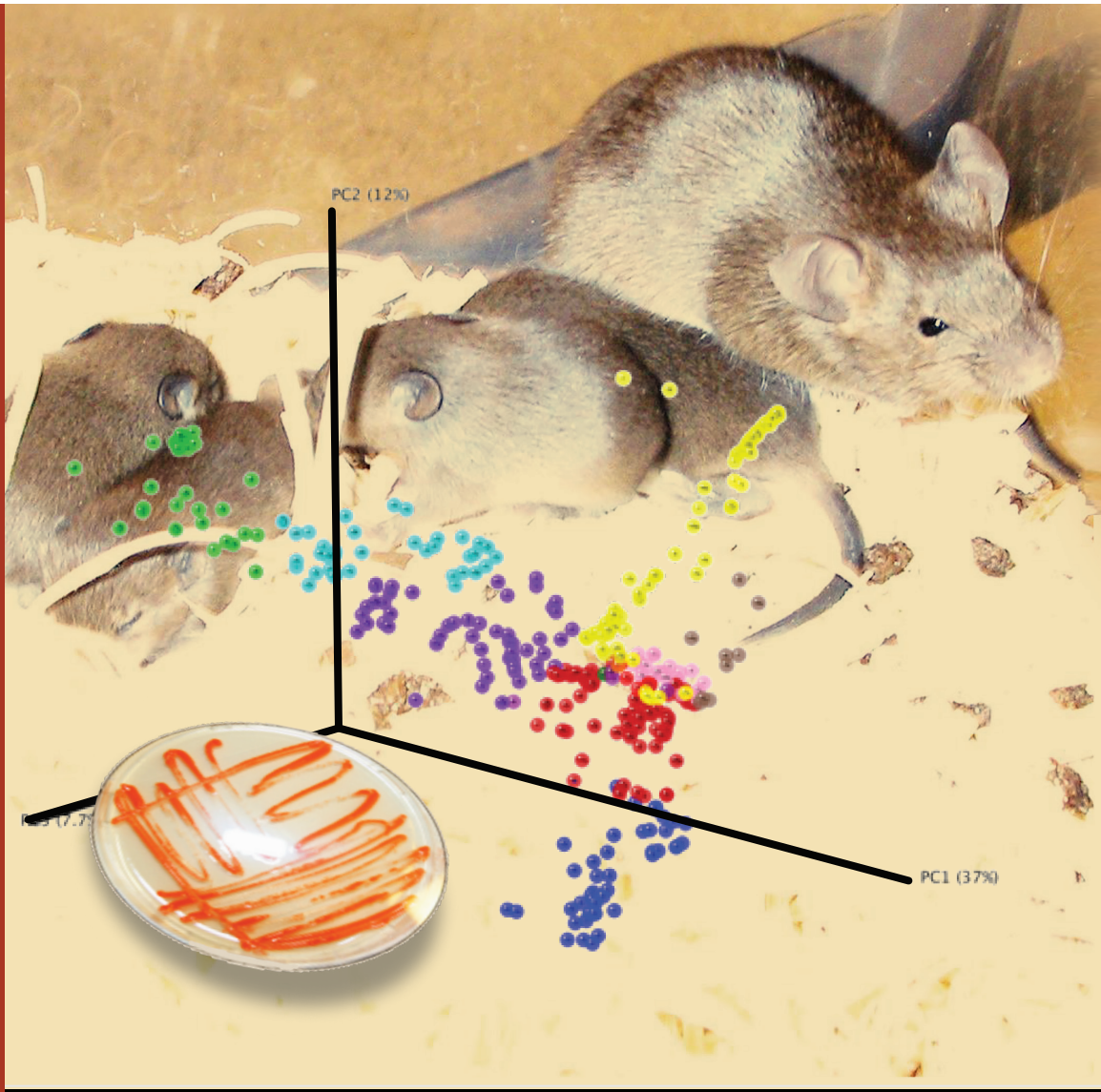


SECOND EDITION

Handbook of Laboratory Animal Bacteriology

Axel Kornerup Hansen
Dennis Sandris Nielsen

CRC Press
Taylor & Francis Group

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Preface

When the first edition of *Handbook of Laboratory Animal Bacteriology* was published in 2000, it was after a decade during which more formal standards, practices, and guidelines had made bacteriological examination of laboratory animals an important part of securing the quality of these animals for research purposes. At the millennium, there was an increasing common understanding on which agents to look for, while the methods on how to do it differed significantly between laboratories. None of the guidelines released during the 1990s gave any detailed advice on how to perform bacteriological study in laboratory animals. Molecular biology was in its very beginning within bacteriology, and cultivation was the tool applied by all laboratories. The better ones among these would be able to cultivate maybe 15–20% of the bacterial species present in an animal; therefore, those organisms listed in quality assurance programs were those that could be cultivated. Today, molecular biology is in many contexts taking over as *the* tool within bacteriology, and in our labs, molecular biology in some form covers most of what we do. This has shown us that laboratory animals of today harbor a range of so far noncultivable organisms with a much larger impact on animal models than could ever be expected from some of those isolated by simple cultivation techniques. This knowledge, however, is still to be recognized among laboratory animal vendors and health-monitoring laboratories, but we hope that with this book we can broaden the understanding on which bacteria make a difference when modeling human diseases in animals and how we should characterize their presence in the animals.

The first part of the book provides information on the general aspects of bacteriology and how to sample and identify bacteria in the animals; as something new, we have tried to give brief insight into how these bacteria interfere with our animal models and the methods used to investigate this. In the second part of the book, the various bacterial species to be found in laboratory rodents and rabbits are described. While in the first edition this was divided according to cultivation and Gram staining characteristics, which was the standard then, it is now divided according to a more systematic phylum-based approach, which the new molecular

biological methods have made standard. This means that bacterial species previously regarded as closely related now may appear in two different phyla. Also, these chapters cover a range of bacteria identified to be of importance in laboratory rodents and rabbits over the past 10–15 years.

We wish to express our sincere thanks to all those master's and doctoral students, postdoctoral students, and laboratory technicians who over the years have helped us to perform all these procedures. Especially, we are grateful to the two young investigators, Lukasz Krych and Camilla Hartmann Friis Hansen, who in our labs have been the driving forces in describing the impact of bacteria on animal models of human diseases. Last but not least, we want to thank our wives, Inge and Mette, for not being too annoyed over the nights we spent writing this book.

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About the Authors

Axel Kornerup Hansen graduated in 1985 as a doctor of veterinary medicine (DVM) from the Royal Veterinary and Agricultural University in Copenhagen, Denmark. After two years in a small-animal hospital, he became head of laboratories at Møllegaard Breeding Center, today part of Taconic Limited. In 1993, he became associate director at the Department of Experimental Medicine, Faculty of Health Sciences, University of Copenhagen. In 1996, he was awarded the degree of doctor of veterinary science based on his thesis on the impact of bacteria in laboratory rats. Since 1997, he has been a professor in laboratory animal science and welfare at the University of Copenhagen (formerly Royal Veterinary and Agricultural University). In 2000, he was made charter diplomat and together with six others founded the European College of Laboratory Animal Medicine. His research area has primarily been on how the microbiota has an impact on laboratory animal models for inflammatory diseases and how this works in conjunction with the diet. He has published 157 peer-reviewed papers in scientific journals and 36 other works in books, proceedings, or popular journals.

Dennis Sandris Nielsen graduated in 2002 with an MSc in food science from the Royal Veterinary and Agricultural University in Copenhagen, Denmark. During his PhD studies (awarded 2006) and postdoctoral years, he has specialized in microbial ecology, with a special interest in gut microbiota, indigenous African fermented foods, and fermented products contributing to the more joyful side of life (cocoa, wine, coffee). This work was obviously not done alone and involved longer and shorter research stays at Max Rubner Institute, Karlsruhe, Germany; Manchester Metropolitan University, United Kingdom; Université de Abidjan, Cotonou, Benin; Food Research Institute, Accra, Ghana; and Cocoa Research Institute of Ghana, New Tafo, Ghana. In 2010, he was appointed associate professor in the Department of Food Science, University of Copenhagen,

with special responsibilities concerning gut microbiota and probiotics; he now heads a research group mainly focused on microbe–host and microbe–microbe interactions in the mammalian gastrointestinal tract. He has published 69 peer-reviewed papers in scientific journals and more than 15 other works in books, proceedings, or popular journals.

chapter one

Laboratory animal bacteriology

The past, the present, and the future

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1.1 Foundation of the discipline of bacteriology

The French chemist Louis Pasteur, the German biologist Ferdinand Cohn, and the German physician Robert Koch are seen as the three main founders of the discipline of bacteriology in the late nineteenth century. Robert Koch is mostly known for his four "Koch's postulates"; that is, to be regarded as a pathogen, a microorganism must be

1. found in abundance in all organisms suffering from the disease but should not be found in healthy organisms.
2. isolated from a diseased organism and grown in pure culture.
3. a cause of disease when introduced into a healthy organism.
4. reisolated from the inoculated, diseased experimental host and identified as identical to the original specific causative agent.

Koch rather quickly disregarded his first postulate because he realized the existence of latent subclinical infections,[1] which in laboratory animal bacteriology today is also the most common condition for bacterial

infections. The second and the fourth postulate can be questioned as well because many infections (caused by, e.g., *Spirillum minus*, which for years has been known as a potential cause of rat bite fever) cannot be cultivated. What is left today is the third postulate, which still seems to have some validity when discussing primary pathogens, such as *Salmonella*, but also is under heavy pressure because many bacteria act in conjunction with other agents. Additionally modern techniques have been used to show that diseases such as diabetes and colitis not primarily seen as infections are also under strong bacterial impact (e.g., from bacteria such as *Akkermansia muciniphila*, which may act protectively against some diseases[2] and induce other diseases[3]). In general, the postulate works poorly for complex pathogeneses, such as those of cancer viruses.[4] On the other hand, Koch's third postulate should not be fully disregarded; that is, an agent must be proved to make some sort of difference in the host organism if it is to be regarded as causative of a pathophysiological condition.

Robert Koch was also the first one to use a solid medium for bacterial cultivation by adding albumin, sterilized vegetables, or starch pastes to gelatinized meat extracts.[5]

1.2 The need for securing the absence of zoonoses

The rat was the first animal to be domesticated for scientific purposes, and the papers on the use of rats for research increased in the second half of the nineteenth century. The alternative to the use of domesticated rats were wild trapped rats, which all were infested with fleas. This presented a risk of spreading pest bacilli, at that time known as *Bacillus pestis*, later as *Pasteurella pestis*, and today as *Yersinia pestis*, and therefore such rats needed to be decontaminated with hazardous chemicals, such as Pinch's oil gas.[6] Other hazardous zoonoses brought in from the wild were *Leptospira*, *Salmonella*, and *Streptobacillus moniliformis*, for example. These were among the first bacterial infections to be eradicated from laboratory rat colonies, but as late as the 1950s reports appeared on laboratory rats infected with *Leptospira*,[7] while *Streptobacillus moniliformis* was isolated from laboratory rats and mice as recently as the 1990s.[8,9] One important need within laboratory animal bacteriology in the early ages of this discipline was therefore securing the absence of such organisms.

1.3 Eradication of bacterial pathogens

1.3.1 The early age of laboratory animal pathology

In 1913, Theobald Smith at the Harvard Medical School in the Boston area described pneumonia in guinea pigs caused by infection with *Bacillus bronchisepticus* (M'gowan),[10] today known as *Bordetella bronchiseptica,* and

in 1917, Ernest Edvard Tyzzer, also at the Harvard Medical School, visualized that a fatal disease with liver necrosis in mice was caused by a long, slender rod that he named *Bacillus piliformis*.[11] The disease hereafter became known as Tyzzer's disease, and when molecular biology enabled further description of the agent, it was renamed *Clostridium piliforme*.[12] A few other works from that time described bacterial infections in research animals. These were, however, few, and rodents still died from infectious diseases in research facilities for decades, but the pioneer works of Smith and Tyzzer showed that there was by then some understanding that certain infections were hazardous for the animals, leading to high losses and thereby reducing the number of animals available for investigations. For many such diseases, the problems were not solved in a preventive manner, but diagnosed, often simply by morphological pathology, when occurring. Animal welfare did not seem to be the key issue until the 1950s, when there arose an increasing understanding that fewer animals should be used in experiments, and the fact that some animals suffered and died for other-than-experimental reasons, such as infectious diseases, was not acceptable. This culminated with the book, *The Principles of Humane Experimental Technique*, by William Russell and Rex Burch in 1959, which introduced the principles of reduction, refinement, and replacement, known as the 3Rs.[13]

1.3.2 Specific pathogen-free animal breeding and health monitoring

Very much in the new spirit of the 3Rs, Henry Foster, who in 1947 had founded a breeding company in Boston and named it after the Charles River, in the late 1950s reported the use of cesarean section and barrier protection as a way to produce specific-pathogen-free (SPF) rodents on a large scale,[14] which also started a new era of commercial breeders supplying quality animals for research rather than the previous in-house breeding colonies with all their infections and infestations. As "old enemies" were eradicated, new bacteria, such as *Citrobacter rodentium* (by then *C. freundii* type 4280),[15] described by Stephen Barthold from the University of California at Davis, were discovered as causative agents of problematic disease outbreaks, in this case transmissible colonic hyperplasia in mice,[16] and eliminated from the breeding colonies. Another new pathogen was *cilia-associated respiratory (CAR) bacillus,* which was isolated by James Ganaway from the National Institutes of Health in Maryland in 1984; this was described as the cause of chronic respiratory disease in rats, characterized as some sort of *Flavobacterium* or *Flexibacterium*,[17,18] and eliminated from infected colonies before fully systematized.[19] Today, only a few of the bacterial agents commonly observed in the 1950s are actually found during routine screenings of rodent colonies.[20–22]

1.3.3 New agents with a research-interfering potential

Through the 1970s and the 1980s, conventional breeding companies were all outcompeted by breeding companies producing only barrier-protected rodents subjected to a thorough health-monitoring program. The response to finding any agents on the list in the beginning was to report them, but hard competition in the long run closed down this option and made the breeding companies eradicate these unwanted bacteria from their colonies. In the early years, it was very much up to the company to decide what to screen for, but in Europe the first set of common standards was introduced in 1994 by the Federation of European Laboratory Animal Associations.[23] This speeded up the process of pathogen eradication. However, as the more severe pathogens were eliminated, new ones with a more discrete impact on the research models would appear. In the 1990s, James G. Fox from the Massachusetts Institute of Technology isolated and characterized a number of well-defined species of *Helicobacter* from several rodent species. Most of them were not linked to specific pathology, but some (e.g., *H. hepaticus*) seem to be able to cause liver tumors in mice.[24] This illustrates how laboratory animal medicine had now developed from dealing with lethal diseases killing entire colonies to dealing with more discrete diseases interfering with the application of the animals as models for human diseases.

1.3.4 The development of health monitoring

Along with the protective types of animal breeding, health monitoring that is more systematic developed. In the 1960s, bacteriology was seen as more or less synonymous with cultivation, which was the main means of diagnostics for bacteria. Bacteria, such as *C. piliforme* (by then *B. piliformis*), that could not be cultured were screened by pathology of target organs. For viruses, cultivations were not a diagnostic option; therefore, serological screening for antibodies was developed and refined from early methods, such as the complement fixation assay evolved to more modern solid-phase assays such as the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), which today seem to be replaced by high-throughput multiplex assays. For some bacteria, this was also the case. In the early 1970s, for instance, it was published that IFA could be used to diagnose *C. piliforme* in infected mice,[25] which through the 1980s became the routine practice in some health-monitoring programs.

This revealed that a large number of European rat colonies apparently seemed to be infected,[26] which gave rise to some debate. On the one side, it called for a new way of thinking among laboratory animal breeders, who had to face that, for some diseases, improvement in production conditions had removed some of the environmental inducers, while the agents were still subclinically present in their colonies. On the other side,

microbiologists had to realize that serology for bacteria carrying a wide range of antibody-inducing epitopes caused far more cross-reactions than viral serology. For an agent such as *C. piliforme,* there seemed to be a true difference between Europe and North America, as many European rat-breeding colonies had been started on the basis of infected reference colonies; furthermore, an agent capable of producing extremely resistant spores would obviously prove difficult to eliminate from the physical surroundings. As the issue of oral tolerance for gut bacteria was never considered (i.e., the fact that the host develops a tolerance toward gut bacteria colonizing in early life preventing, among other things, the formation of systemic antibodies), it seemed much easier for all parties to accept the fact that some agents (e.g., CAR bacillus) were only rarely diagnosed by serology and could be regarded as a true expression of reality.[20]

Although an agent such as *Helicobacter*, which was first described in laboratory rodents in the 1980s, can be cultivated, the introduction of the less costly and less laborious polymerase chain reaction (PCR) made this the method of choice for screening of such new agents,[27] and, therefore, for *Helicobacter*, cultivation techniques never really found a place in routine health monitoring. Although cultivation is still used today as the preferred tool for bacterial screening, PCR or other similar molecular methods will be replacing it for most bacterial infections in future health monitoring of rodent colonies.

1.4 The impact of the symbiotic microbiota

1.4.1 The development of gnotobiotechnology

The rodent gastrointestinal tract (representative of the case for all other mammals) is inhabited by more than 500 species of symbiotic bacteria, depending on how they are identified and categorized. One of Louis Pasteur's hypotheses was that animal life without indigenous microorganisms would be impossible[28]; two other key scientists of that time, Marceli Nencki and Élie Metchnikoff, on the contrary stated it would be longer and healthier.[29,30] To prove either one of these hypotheses from the late nineteenth century until around 1950, eight different groups made attempts to produce germ-free animals.[31] Around the time of World War I, it was clear that animals could live without germs for extended periods, and in the 1930s, Gusta Glimstedt and Bengt Gustaffsson at the University in Lund in Sweden and James A. Reyniers at the University of Notre Dame in Indiana reported the rearing of germ-free animals in stainless steel isolators, thereby developing the discipline of gnotobiotechnology.[32,33] The first screenings of germ-free status were based solely on cultivation and microscopy, but there seemed to be a clear understanding that there might be other microbes present in an abun-

dance too low to allow detection by these means.[34] Today, germ-free rearing is common practice in a range of laboratories all over the world.

1.4.2 Schaedler's flora

Germ-free rodents seem to be predisposed to opportunistic infections when removed from their isolators and introduced into barrier rooms. As just a few bacteria seem to minimize this phenomenon, Russell W. Schaedler from Thomas Jefferson University in Pennsylvania in 1965 isolated and grew bacteria from conventional and SPF mice housed at Rockefeller University in New York.[35] What he found were categorized as lactobacilli, streptococci, *Flavobacterium* spp., coliforms, enterococci, and *Bacteroides* spp.[35] He selected some of the more dominant bacteria that could be isolated in culture, such as two strains of lactobacilli (thought to be *Lactobacillus acidophilus* and *L. salivarius*), one strain of anaerobic streptococci group N (*Streptococcus fecalis*), two strains of *Bacteroides* spp. (one of them thought to be *Bacteroides distasonis*), and one coliform strain (*Escherichia coli).* Subsequently, he fed some of these bacteria to germ-free mice.[36] Germ-free mice associated with this combination could be bred for generations still harboring the flora, and in contrast to germ-free animals, which exhibit a severely enlarged cecum and abnormal intestinal histology, the intestine and cecum of the recolonized mice and their offspring appeared entirely normal.[36] In other combinations, he included a species of *Clostridium,* along with an anaerobic fusiform species,[37] and the cocktail became known as the Schaedler flora.

In 1978, one of his former doctoral students, Roger P. Orcutt, helped the National Cancer Institute in Maryland revise and standardize the Schaedler flora, making it the altered Schaedler flora (ASF).[37] The two lactobacilli, the *Bacteroides*, and the fusiform bacterium remained in the new cocktail, and four new bacteria were added.[37] Precise methods for confirmation and characterization did not come along until later, but today the ASF bacteria are characterized as shown in Table 1.1.[37] The ASF have become the starting flora of many breeding colonies. In the beginning, these were seen as colonization-resistance floras (i.e., it was hoped that they would prevent other microbes from colonizing). However, the majority of the remaining members of the microbiome of a barrier-bred colony are most likely transferred from either humans emitting several microbe-carrying particles per minute[38] or from the diet.[39] Therefore, such a phenomenon as normal flora probably does not exist for these animals because little of it originates from the natural habitat of mice. In two different studies, SPF mice showed a less-complex flora, fewer *Bacteroides* and *Lactobacillus* spp., but more *Clostridia* spp., when compared to either feral or conventional mice, and although similar bacteria were detected, the ASF bacteria were not recovered.[40,41] Other groups, however, have

Table 1.1 Bacterial Species in the Altered Schaedler Flora

Original designation	Systematics
ASF 356	Closely related to *Clostridium propionicum*
ASF 360	Novel *Lactobacillus* spp. clustering with *L. acidophilus* and *L. lactis*
ASF 361	*Lactobacillus* spp.
ASF 457	*Mucispirillum schaedleri*
ASF 492	*Eubacterium pexicaudatum*
ASF 500	A novel unnamed genus in Firmicutes with Bacillaceae and Clostridiaceae relations
ASF 502	*Clostridium* cluster XIV
ASF 519	A novel unnamed genus in Bacteroidetes clustering with *B. distasonis*, *B. merdae*, CDC group DF-3, and *B. forsythus*.

Source: Dewhirst FE, Chien CC, Paster BJ, Ericson RL, Orcutt RP, Schauer DB, et al. *Appl Environ Microbiol* 1999; 65:3287–3292.

observed the colonization of the ASF bacteria even several years after establishing a barrier unit,[42,43] but also that the profile is subject to a substantial cage effect.[43]

1.4.3 Microbiome studies

Cultivation today still seems to be the main practice for screening for germ-free status, maybe because more modern techniques based on molecular biology show too much contamination. However, while the first gnotobiotechnologists were unable to describe more than approximately 15–20% of what could be of potential interest in the microbiome, modern sequencing techniques have allowed full characterization of the microbiome. This has led both to studies in which microbiota-harboring mice have been characterized in relation to the development of specific human diseases, such as type 1 diabetes,[44] type 2 diabetes,[45] obesity,[46] and colitis,[47] and to a new age of germ-free studies in which germ-free mice are recolonized with a microbiota of other mice with a specific phenotype hypothesized to be transferred with the microbiota. One of the pioneers of this has been Jeff Gordon from Washington University, and it was in his laboratory that Peter Turnbaugh in 2006 transferred the obese phenotype of leptin-deficient mice to germ-free wild-type mice in isolators.[48] Today, this has become some sort of revival of Koch's third postulate, that is, to show that a certain phenotype is under impact of the microbiome, gut contents are transplanted to germ-free mice, which afterward are characterized for traits of that phenotype. An alternative approach has been to turn off a specific phenotype by the application of antibiotics.[49–52]

1.5 The future of laboratory animal bacteriology

One of the older bacteria still found in rodent colonies is *Pasteurella pneumotropica*. In 1969, Patricia Brennan showed that it could only induce pneumonia in mice if inoculated together with *Mycoplasma* spp.[53] However, for many years, it was considered a primary inducer of suppurative disease in mice.[54] Along with the evolvement of screenings for viruses and *Mycoplasma* spp., breeders have eliminated these primary pathogens from their colonies, thereby also minimizing previous problems related to *P. pneumotropica*, which obviously has been mostly secondary to other agents. The reason for *Pasteurella*'s "success" as a member of health-monitoring programs is probably because of the combination of its ability to be cultivated and its difficult eradication rather than its impact

Table 1.2 Five Examples of Bacterial Species Newly Recognized for Their Potential to Interfere with Animal Models and Therefore Potential Candidates for Future Membership of Bacteriological Screening Programs

Species	Phylum	Impact on rodent models
Segmented filamentous bacteria (SFBs) or *Candidatus savagella*	Firmicutes	Increased severity of inflammatory bowel disease[1] Decreased incidence of type 1 diabetes[2]
Bifidobacterium spp.	Actinobacteria	Decreased severity of inflammatory bowel disease[3–6] Decreased level of inflammation[7] Decreased level of leptin in rats[8] Decreased allergic sensitization[9] Decreased myocardial infarction[8]
Bacteroides vulgatus	Bacteroidetes	Increased severity of inflammatory bowel disease[10]
Bacteroides fragilis	Bacteroidetes	Increased severity of inflammatory bowel disease[11] Increased incidence of colonic tumors[12] Protection against *Helicobacter hepaticus*-induced colitis[13]
Akkermansia muciniphila	Verrucomicrobia	Decreased severity of obesity and type 2 diabetes[14,15] Decreased incidence of type 1 diabetes[16] Increased susceptibility to allergic asthma[17] Decreased severity of inflammatory bowel disease[18] Higher-severity *Salmonella typhimurium* infection[19] Increased incidence of colonic tumors[20]

Table 1.2 (continued) Five Examples of Bacterial Species Newly Recognized for Their Potential to Interfere with Animal Models and Therefore Potential Candidates for Future Membership of Bacteriological Screening Programs

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on research. Today, as PCR is replacing cultivation as a diagnostic tool, what can be cultivated becomes less relevant and which agents are likely to change the expression of research models more relevant.

Table 1.2 provides some examples of bacterial species for which modern techniques based on PCR or sequencing have revealed a strong potential for influencing animal models.[3,45,55–72] Many of these seem to have a broader and far more serious impact on animal models than many easily cultivable bacteria. Therefore, it is likely as costs of sequencing and multiplex PCRs are rapidly declining that these bacteria will be the future members of routine bacteriological programs in rodent colonies rather than "old enemies" such as *P. pneumotropica*.[73] Their absence will not be the only goal. For some models, they will act in favor of the model; for others, they will act against it. Not only their qualitative association with

the animals (i.e., whether they are to be found or not in the animal) will be of interest but also it will be necessary to consider their abundance. It is also reasonable to assume that a full microbiome characterization in the future will become an important part of the characterizations of many animal models.

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chapter two

Sampling animals for bacteriological examination

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2.1 Examining healthy animals

It is essential that rodents used for biomedical research are *microbiologically defined* (see also Chapter 6), meaning that the microbiological status of the animals used is known. However, as evaluation of the microbiology of the individual animal taking part in the experiment is difficult, these factors generally will be monitored in animals sampled only for this purpose, and status will be a picture of the population, not the individual. For example, a certain rat colony may be infected with *Salmonella*, which means that there is a certain risk that rats from this colony may develop salmonellosis during the experiment. However, it does not mean that the rats in the experiment necessarily carry *Salmonella* and that they will

develop salmonellosis, only that there is a certain risk that they will. Thus, the microbiological status is generally given as a list of microorganisms monitored within the colony, with a designation regarding whether they have been found, without any remarks concerning the organisms causing disease.

Revealing a pathogen-free status has traditionally been done by routine sampling of healthy animals from the colony. To reveal bacteria with a certain research-interfering potential, samples are taken from these animals. The bacteria are normally not in a state to cause disease in that animal and are therefore not found as pure cultures in the animal. Therefore, bacteria of interest have to be identified among many other bacteria (of no interest) also found in the animal.

How many animals to sample, how often to do it, what to look for, and which methods to choose should be based on scientific judgment dependent on the type of research and animals involved, but international guidelines may be consulted. In Europe, a set of recommendations has been issued by international organizations within laboratory animal science, such as the Federation of European Laboratory Animal Science Associations (FELASA).[1] Table 2.1 shows which bacteria these guidelines recommend for routine health monitoring. The guidelines give only sparse information on methodology for identifying bacteria, but it is stated

Table 2.1 Bacteria to Be Included in Health-Monitoring Programs According to the FELASA Guidelines for Health Monitoring of Rodents and Rabbits

	Mice	Rats	Hamsters	Guinea pigs	Rabbits
Bordetella bronchiseptica	−	+	−	+	+
Cilia-associated respiratory bacillus	−	+	−	−	+
Citrobacter rodentium	+	−	−	−	−
Clostridium piliforme	+	+	+	+	+
Corynebacterium kutscheri	−	−	+	+	−
Helicobacter spp.	+	−	+	−	−
Mycoplasma spp.	+	+	−	−	−
Pasteurella pneumotropica	+	+	+	+	−
Pasteurella multocida	−	−	−	−	+
Salmonella	+	+	+	+	+
Streptobacillus moniliformis	+	+	−	+	−
β-Hemolytic streptococci	+	+	−	+	−
Streptococcus pneumoniae	+	+	−	−	−

Source: Mähler M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, et al. FELASA Working Group on Revision of Guidelines for Health Monitoring of Rodents and Rabbits. *Lab Anim* 2014 March 4. [Epub ahead of print]

that "culture techniques are usually employed for the detection of most bacterial and fungal agents."[1] The scope of this book is to provide precise details of how this kind of examination may be performed, as well as providing a broader picture of the "normal" microbiota in laboratory (rodent) animals.

2.1.1 Sampling strategies

2.1.1.1 Random sampling

Three basic premises are adhered to when screening larger colonies of laboratory animals for specific agents[2]:

1. A few animals can be sampled for examination, but the results can be used to describe the entire colony.
2. If one animal is found to be infected with a certain organism, the entire colony is considered infected with that particular organism.
3. If no animals are found to be infected with a certain organism, the entire colony is considered free of that particular organism.

Principle 1 presupposes independence between the animals sampled. One can define the group to sample; for example, it is known that *Akkermansia muciniphila* is only found in young leptin-deficient mice,[3] so all animals sampled should be of that age. Sensu strictu, the results will only be valid for animals of that age, but it is assumed that if the agent is not found in animals of the specified age, it is not to be found at all. When one or several of such criteria have been defined, it is of vital importance to sample among the animals fulfilling these criteria in a way that avoids the influence of other criteria (e.g., animals must not be sampled from the same cage, from the same end of the unit, etc.). If such independence claims are not fulfilled, the conclusion cannot be extended to cover all animals within the unit.

2.1.1.2 Calculation of the sample size

The fraction of animals in a colony infected at a certain moment is termed the *prevalence* (*p*). The prevalence that a certain infection reaches depends on many factors (e.g., the contact between the animals, the resistance of the animals, etc.). However, characteristics of the agent itself play a major role. It is the experience in laboratory animal epidemiology that the observed prevalence of a certain agent usually falls within a certain range.[4] As far as the colony sampled from is so large that sampling itself does not affect the prevalence, it can be assumed that the prevalence is independent of the population size. This equilibrium is reached around a colony size of approximately 1000. If all the infected animals, and only the infected

animals, in a population react positively in a given test system, then the risk of reaching a false-positive diagnosis C by sampling one animal is

$$C = 1 - p \tag{2.1}$$

while the sampling of S animals gives

$$C = (1 - p)^S \tag{2.2}$$

in which S is the sample size. This gives the equation for the sample sizes normally used for health monitoring in colonies of laboratory animals[5]:

$$S \geq \frac{\log C}{\log(1 - p)} \tag{2.3}$$

If the examination is to have more than 95% probability of being correct, the *confidence limit* C is 0.05. If certain criteria, as described, are outlined prior to sampling, the prevalence to be used is the prevalence in the group sampled from and not the overall prevalence in the colony.

Equation 2.3, however, is too simplified. When using the term *prevalence*, it is assumed that this is the *real prevalence*, which means the number of animals actually infected with the organism. However, in reality it does not matter how many animals are infected, only how many react positively in the test system used. For example, if no infected animals react positively in the system, then the chance of reaching a false diagnosis would be 100%.

Based on Table 2.2, the following equations can be made[6]:

$$\text{Sensitivity} = a/(a + b) \tag{2.4}$$

$$\text{Specificity} = d/(c + d) \tag{2.5}$$

Table 2.2 Definition of the Terms

	Test result	
	Positive	**Negative**
Infected	a	b
Not infected	c	d

Note: (a) True positives; (b) false negatives; (c) false positives; (d) true negatives.

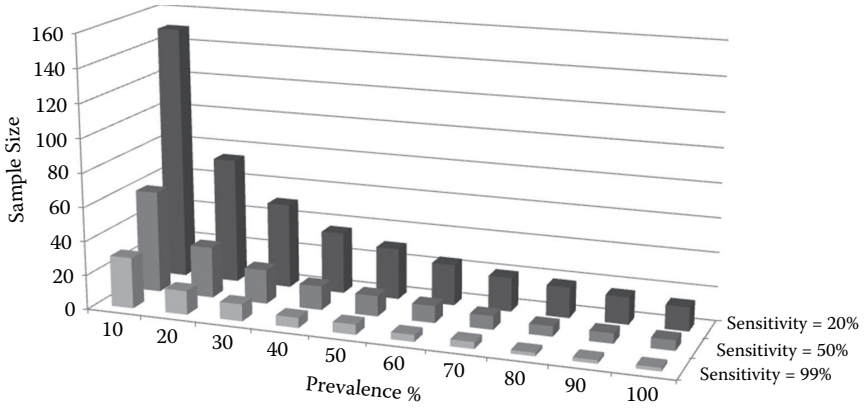


Figure 2.1 The correlation between sample size and expected prevalence of a certain infection at different sensitivities. The risk of a false-negative result is 0.05 for all bars.

The prevalence to be used for calculation of the sample size is only that fraction out of the real prevalence p that also will react positively in the test, that is, $p \cdot Sensitivity$, which leads to the following equation[2]:

$$S \geq \frac{\log C}{\log\left(1-\left(p \cdot \text{Sensitivity}\right)\right)} \tag{2.6}$$

The relationship is shown in Figure 2.1.

2.1.2 Sampling frequency

A sample visualizes the status at the moment of sampling. As soon as it has been taken, it becomes historical. Only curiosity will dictate when to take the next sample. If two samplings taken at a certain interval are compared, the last sampling will visualize whether changes have occurred between the two samplings. It could be argued that if 50 animals must be sampled to reach a confidence limit above 95%, and 1 animal is sampled every week, the confident sample size is reached after a year. The calculation may be correct, at least for persisting infections, but what has been calculated is a 95% confidence of the status a year ago.

2.1.3 Defining the microbiological entity

It is rather obvious that a random sample of animals may only be predictive for the microbiological status of other animals if these animals have some kind of contact with one another. Animals of the same strain and

species differ in their microbiota if kept in separate rooms or just in different cages.[7] Even different routine practices (e.g., in relation to cage changing) may lead to differences in the microbiota.[8] Therefore, it is essential to define the microbiological entity, that is, provide a definition of the group of animals for which a sample is predictive. This is a complicated matter.

Isolators and each cage in an individually ventilated system should be defined as separate microbiological entities because the idea of the system is to keep the animals out of contact with the surroundings, but because of the limited space, there is close contact between the animals. Likewise, a simple one-room unit used for breeding at a commercial vendor will usually have its own staff, routines, and a barrier to separate different units from one another; therefore, each of these separate units should be regarded as separate microbiological entities. However, if the unit is separated into different rooms, all of which are served by the same staff and supplied from a common barrier, it is difficult to define the microbiological entity. Some bacteria easily spread from room to room, and others do not. The gut microbiota is to a large extent established around weaning and because of the oral tolerance generated by the immune system is difficult to change later in life.[9]

Each experiment consists of several cages, each room may contain several experiments, and behind each barrier there may be several rooms. The correct level to define the microbiological entity is not a simple matter, but it should be noted that the more subunits to be included, the higher the insecurity of the sampling; for example, if the infection is only found in one subunit, the prevalence is "diluted," which may not be taken into consideration when calculating the sample size.

2.2 *Characterization of the commensal microbiota*

In routine bacteriological monitoring, the primary aim is to verify the presence or absence of certain specified bacteria with a potential of either pathogenicity or research interference. This is called *screening*. If a dichotomic identification leads to a path that does not contain any of those bacteria screened for, any further identification may be omitted. However, in the last decade, possibilities of describing the microbiological variation have increased substantially, and it has been shown that this microbial variation to a large extent is responsible for the variation shown in animal models.[10] Therefore, in some studies there is the need to identify all microorganisms (or at least bacteria) present as precisely as possible. This process is called *profiling*.

Such analysis of the microbiota started with the culture of aerobic and facultative anaerobes[11,12]; later, it also became possible to culture members of the anaerobes, which in the gut represent 99% of the bacteria.[13,14] However, only 10–20% of the bacterial species residing in the gut

can be cultivated by classical techniques[4,15–19]; therefore, although having had some application in various types of research (e.g., within type 1 diabetes),[18] the outcome of profiling seldom balanced the workload of performing it. This has changed over the past two decades with the development of methods based first on gas chromatography, then on polymerase chain reaction (PCR). Fluorescent *in situ* hybridization (FISH), quantitative PCR (qPCR), and now high-throughput sequencing have exploded and allowed rapid and precise microbiological profiling of the gastrointestinal ecosystem.[7,10,20–38] For some years, it has been common to characterize the variation in a group or differences between groups by electrophoresis of PCR-propagated microbial genomic material,[7,21,22,24,28] which will describe the variation and the differences without actual identification. A further development has been the application of multiple qPCRs for making a qualitative as well as quantitative profile of predefined species and phyla.[39] High-throughput sequencing, which until recently was more costly, has been used subsequently to reveal the qualitative nature of the profile; today, for many studies, sequencing without any supplementary methods seems to be the choice for profiling.[35–38]

For profiling of any microbiota, depending on the target organisms, it is not necessarily essential to use an anaerobic chamber. However, if strict anaerobes are targeted or the sample is to be used for inoculation transfer into other animals, it may be beneficial to sample from the gut inside an anaerobic chamber, to which the animal must be transferred for sampling immediately after euthanasia.

2.3 Sampling procedures

2.3.1 Planning the work

A full bacteriological investigation includes several procedures that might be time consuming (i.e., because the samples at various steps have to be incubated overnight or for a number of days). Therefore, the work has to be carefully planned. For routine sampling, this may be done as proposed in Table 2.3.

2.3.2 Euthanasia

If the only procedures to take place are cultivation or molecular biological characterizations, the animal will not have to be alive during sampling, so it should be humanely killed prior to the procedure. It is essential that this killing does not interfere with sterility or destroy the organs to be sampled. For small rodents and guinea pigs, the safest way to ensure this is by injecting approximately 2 ml/kg body weight of sterile 10% pentobarbitone mixed up in ethanol together with lidocaine intraperitoneally. This

Table 2.3 Example of a Working Plan for Isolation, Cultivation, and Identification of Bacteria from Laboratory Animals

Monday	Sampling and inoculation from the animals			
Tuesday	*First reading of primary plates* Subcultivation of cultivated bacteria			Subcultivation from some selective enrichment broths onto solid media
Wednesday	Tests for dividing isolated bacteria into major groups or genera Immunological and enzymatic tests Inoculation in test media for identification	*Second reading of primary plates* Subcultivation of cultivated bacteria		*Reading and interpreting of solid media* Subcultivation of cultivated bacteria
Thursday	Reading test media Inoculating supplementary media	Tests for dividing isolated bacteria into major groups or genera Immunological and enzymatic tests Inoculation in test media for identification Extraction of DNA, PCR for sequencing, send amplicons for sequencing	*Third reading of primary plates* Subcultivation of cultivated bacteria	Tests for dividing isolated bacteria into major groups or genera Immunological and enzymatic tests Inoculation in test media for identification Extraction of DNA, PCR for sequencing, send amplicons for sequencing

Friday	Reading supplementary media	Reading test media	Tests for dividing isolated bacteria into major groups or genera Immunological and enzymatic tests	Reading test media
Monday		Inoculating supplementary media Analyze sequences Analyze sequences	Inoculation in test media for identification	Inoculating supplementary media
Tuesday		Reading supplementary media	Inoculating supplementary media	Reading supplementary media
Wednesday			Reading supplementary media	
		Conclusion and reporting		

Note: Often, media must be incubated longer than assumed in this table.

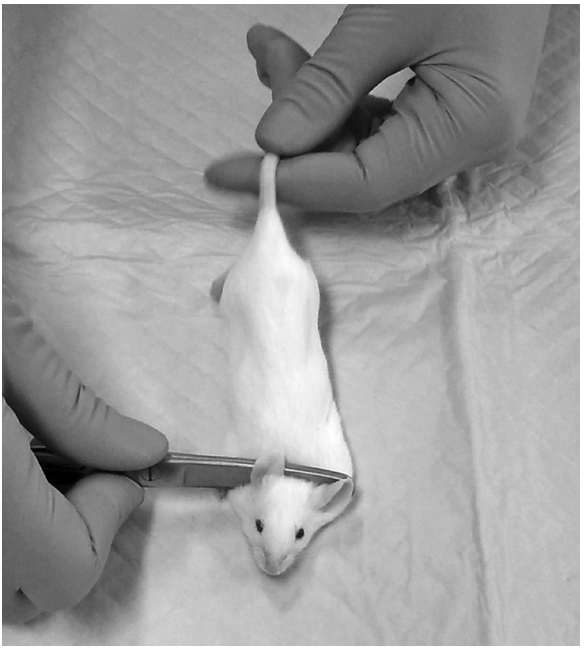


Figure 2.2 Cervical dislocation in a mouse.

method may interfere with abdominal sampling. Alternatively, cervical dislocation, by which the neck is stretched to break both the carotid blood supply and the medulla (Figure 2.2), may be applied. However, breaking the neck will normally destroy the trachea and thereby prevent proper sampling from this site. Larger animals, such as rabbits, should be given the pentobarbitone injection intravenously.

2.3.3 Blood sampling

If blood has to be sampled for serology, the animal should first be anesthetized and then killed. For most rodents, guinea pigs, and rabbits, the preferable anesthetic agent is the combination of fentanyl-fluanisone (Hypnorm®) and midazolam (Dormicum®), which may be used according to Table 2.4. As Hypnorm is difficult to purchase in the United States because of Drug Enforcement Agency regulations, US laboratories may have to search for alternatives, such as a combination of ketamine and either xylazine or acetylpromazine (Table 2.4). For each animal, further information may be found in textbooks on anesthesia (e.g., Flecknell, 2009).[40]

When the animal is sufficiently anesthetized blood is sampled by periorbital puncture (Figure 2.3), heart puncture (Figure 2.4), or another

Table 2.4 Anesthesia of Rodents, Guinea Pigs, and Rabbits with Ketamine (Vetalar®, Ketaset®, Ketalar®, Ketaminol®), Xylazine (Rompun®, Narcoxyl®), and Acetylpromazine (Acepromazine®)

	Dose mg/kg body weight			
Species	Ketamine	Xylazine	Acetylpromazine	Route
Mouse	100	15		Intramuscularly, intraperitoneally
Rat	100	15		Intramuscularly, subcutaneously
Guinea pig	40	5		Intramuscularly, subcutaneously
Hamster	200	10		Intramuscularly, subcutaneously
Gerbil	50	2		Intramuscularly
Rabbit	75		5	Intramuscularly

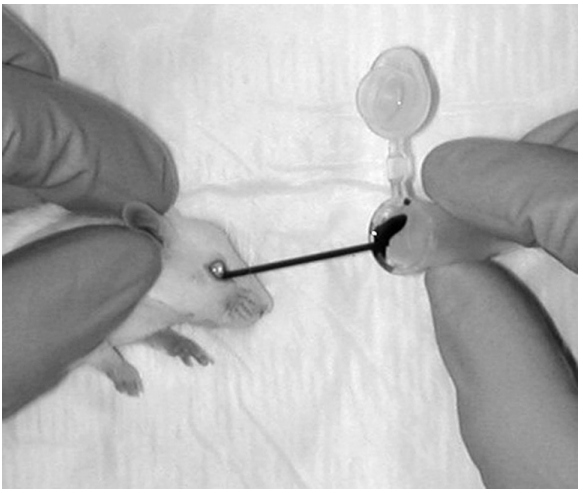


Figure 2.3 Sampling blood from a mouse by periorbital puncture using a hematocrit tube.

satisfactory method. Detailed instructions are given elsewhere.[41] After blood sampling, the animal is killed as described previously.

If serum is used instead of plasma, it is recommended to place a wooden stick with the blood sample in the tube immediately after sampling. The blood sample is then left at room temperature for at least 3 h, after which most of the coagulum can be removed with the stick (Figure 2.5). The remaining coagulum is removed by centrifugation at 2000 to 3000 rpm for 20 min.



Figure 2.4 Sampling blood from a rat by heart puncture using a vacuum blood-sampling glass.

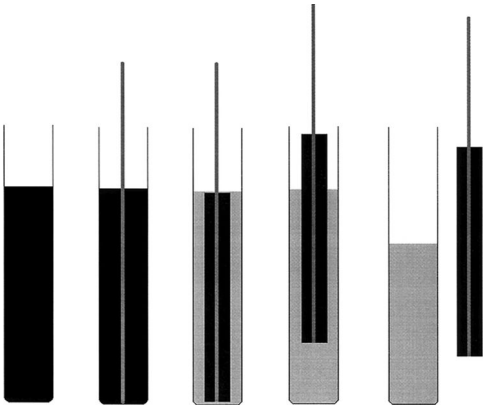


Figure 2.5 Removal of the coagulum from a blood sample might be facilitated by placing a wooden stick with the blood sample in the tube immediately after sampling. The blood sample is then left at room temperature for at least 3 h, after which most of the coagulum can be removed with the stick.

2.3.4 Instruments and sterilizing procedures during sampling

Sampling for culture is done with a platinum needle (e.g., 0.5 mm). The needle is flame sterilized between each sampling. To open the animal and the various organs, it is necessary to have at least a scalpel, a pair of scissors, and a pair of tweezers, which must be sterilized by autoclaving or dry heat before every sampling. Sterilization is achieved by cleaning the



Figure 2.6 Instruments for bacteriological sampling. The instruments are dipped into 70% ethanol in the little glass, and while still wet from the alcohol, they are held into the burning flame and placed on the glass rack until the alcohol has ceased burning. Care should be taken to avoid touching animals disinfected with ethanol with the instruments while the alcohol is still burning.

instruments in a glass of ethanol (70% or 93%) and holding them in a flame between each sampling. The burning instruments are then placed on, for example, a glass rack (Figure 2.6), until the fire has ceased. If available, a glass bead sterilizer (Figure 2.7) may be preferred, placing the tips of the instruments into a well filled with hot glass beads for approximately 2–5 s. For either of the methods, the instruments must be allowed to cool before use.

2.3.5 Opening and inspecting the carcass

The animal is placed on its back on a polystyrene tray, and its four feet are attached to the tray with pins (Figure 2.8). The entire thoracic and abdominal wall may be disinfected with 70% ethanol, which should be allowed to evaporate before any procedures are performed. This disinfection procedure is not 100% necessary for sterile sampling and may be omitted.

2.3.6 Sampling from various organs

If the reason for examination is some kind of disease in the animal, which organ to sample may be rather straightforward. If a healthy animal is sampled for investigations for one or several bacteria, the choice of organs to be sampled must be based on scientific judgment (Figures 2.9 to 2.37). In Tables 2.5 (also Figures 2.9 to 2.20) and 2.6 (includes Figures 2.27 to 2.37),



Figure 2.7 Glass bead sterilizer. Instruments are placed in the opening for a few seconds to sterilize their tips.



Figure 2.8 The animal is placed on its back on a polystyrene tray; its four feet are attached to the tray by pins.

Table 2.5 Bacteriological Sampling Sites Accessible from the Body Surface and Bacterial Species Likely to Be Isolated from These Sites

	Healthy animals	Diseased animals	How to sample
Skin	*Staphylococcus, Pseudomonas* spp.	*Corynebacterium kutscheri, Staphylococcus, Pseudomonas* spp.	The area is scraped with a scalpel, and the scraping is transferred to the medium (Figure 2.9); the material is streaked with the platinum needle on an agar plate (Figure 2.10).
Nose	*Bordetella bronchiseptica, Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumoniae*	*Bordetella bronchiseptica, Klebsiella pneumoniae, Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumoniae*	The skin around the nostrils is removed with scissors (Figure 2.11); the platinum needle is introduced deeply into both sides of the nasal cavity (Figure 2.12).
Conjunctiva	β-Hemolytic streptococci	Pasteurellaceae, *Staphylococcus*, β-hemolytic streptococci	The conjunctiva is touched with the platinum needle (Figure 2.13).
Middle ear	*Mycoplasma* spp.	*Corynebacterium kutscheri, Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., *Staphylococcus*, β-hemolytic streptococci, *Streptococcus pneumoniae*	The entire auricle is cut off with scissors (Figure 2.14); the accessible membrane (Figure 2.15) is opened with a scalpel, and the platinum needle is introduced into the middle ear (Figure 2.16).

continued

Table 2.5 (continued) Bacteriological Sampling Sites Accessible from the Body Surface and Bacterial Species Likely to Be Isolated from These Sites

	Healthy animals	Diseased animals	How to sample
Trachea	*Bordetella bronchiseptica*, *Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumoniae*	*Bordetella bronchiseptica*, *Klebsiella pneumoniae*, *Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumoniae*	The animal is placed on its back, the skin in the ventral neck region is opened, and the muscles over the trachea are bluntly spread (Figure 2.17); the trachea is lifted by placing the scissors underneath it (Figure 2.18), and the trachea is opened with a longitudinal cut (Figure 2.19); the needle is introduced in both the cranial and the caudal directions (Figure 2.20).
Feces	*Campylobacter* spp., *Citrobacter rodentium*, *Mycobacterium* spp., *Pseudomonas* spp., Salmonellae, *Yersinia pseudotuberculosis*	*Campylobacter* spp., *Citrobacter rodentium*, *Pseudomonas* spp., Salmonellae, *Yersinia pseudotuberculosis*	A fecal pellet is dropped into a broth or suspended in 1 ml sterile saline; the suspension is streaked on an agar plate with the platinum needle.

Table 2.6 Bacteriological Sampling Sites Accessible Only after Opening Thorax or Abdomen and Bacterial Species Likely to Be Isolated from These Sites

	Healthy animals	Diseased animals	How to sample
Lungs	*Mycoplasma* spp.	*Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Klebsiella pneumoniae*, *Mycobacterium* spp., *Mycoplasma* spp., Pasteurellaceae, *Pseudomonas* spp., β-hemolytic streptococci, *Streptococcus pneumoniae*	A piece of each of the lungs is cut off with scissors, and the cut surface is pressed into the surface of an agar plate (Figure 2.27); the material is streaked with the platinum needle (Figure 2.10); for propagation, the piece of lung is simply dropped into an enrichment broth.

Table 2.6 (continued) Bacteriological Sampling Sites Accessible Only after Opening Thorax or Abdomen and Bacterial Species Likely to Be Isolated from These Sites

	Healthy animals	Diseased animals	How to sample
Cecum	*Campylobacter* spp., *Citrobacter rodentium, Escherichia coli, Klebsiella pneumoniae*, Pasteurellaceae, *Salmonella, Yersinia pseudotuberculosis, Helicobacter* spp.	*Campylobacter* spp., *Citrobacter rodentium, Escherichia coli, Klebsiella pneumoniae*, Pasteurellaceae, *Salmonella, Yersinia pseudotuberculosis, Helicobacter* spp.	A piece of the cecal wall (major curve, upper side, midsection) is cut off with scissors (Figure 2.28), and the platinum needle is dipped into the cecal contents (Figure 2.29), which are then either streaked on an agar plate or dipped into a broth.
Liver	*Helicobacter* spp.	*Corynebacterium kutscheri, Escherichia coli, Helicobacter* spp., Pasteurellaceae	A lobe of the liver is grasped with the tweezers, and the grasped piece is cut off (Figure 2.34); the piece is plunged into a broth or the cut surface is touched on the agar plate (Figure 2.35), which then is streaked with the platinum needle.
Genitals	*Corynebacterium kutscheri, Escherichia coli, Mycoplasma* spp., Pasteurellaceae, β-hemolytic streptococci, *Streptococcus pneumoniae, Listeria* spp.	*Corynebacterium kutscheri, Escherichia coli, Mycoplasma* spp., Pasteurellaceae, β-hemolytic streptococci, *Streptobacillus moniliformis, Streptococcus pneumoniae, Listeria* spp.	♀: The vulvae are cut off with scissors (Figure 2.30), and the platinum needle is introduced deeply into the vagina (Figure 2.31). ♂: The preputium is cut off (Figure 2.32), and the glans is touched with the platinum needle (Figure 2.33); the needle then is either streaked on an agar plate or dipped into a broth.

continued

Table 2.6 (continued) Bacteriological Sampling Sites Accessible Only after Opening Thorax or Abdomen and Bacterial Species Likely to Be Isolated from These Sites

	Healthy animals	Diseased animals	How to sample
Spleen		Pasteurellaceae, *Streptobacillus moniliformis, Streptococcus pneumoniae*	Same as for the liver
Kidneys		*Corynebacterium kutscheri, Escherichia coli*	The kidney is halved in the pole-to-pole direction (Figure 2.36), and the pelvic surface is touched with the platinum needle (Figure 2.37).



Figure 2.9 An area of the skin to be sampled is scraped with a scalpel, and the scraping is transferred to the medium.

information is given on which organs to sample in the search for certain bacteria. Further descriptions are given for each agent in the specific chapters. Programs should be planned individually according to the aims. For routine monitoring of rodents, guinea pigs, and rabbits, it is recommended to include at least the nose, trachea, cecum, and the genitals in the examination.

The abdomen is opened by first removing a piece of skin with scissors in the longitudinal direction of the animal all the way from above

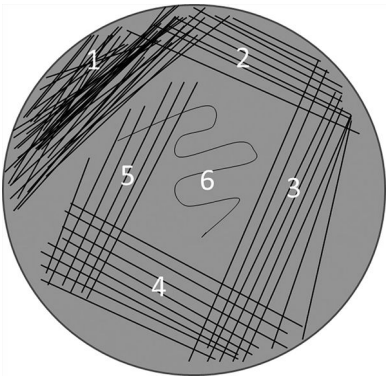


Figure 2.10 Correct streaking of an agar plate.



Figure 2.11 The skin around the nostrils is removed with scissors to facilitate sampling from the nose.

the pecten ossis pubis to the sternal manubrium (Figures 2.21 and 2.22). A piece of the abdominal wall with muscles and peritoneum is removed from the pecten ossis pubis to the manubrium (Figure 2.23). The abdominal organs then can be located and sampled (Figure 2.24). To access the thorax, the skin above the sternum is removed. The thorax is opened by lifting the manubrium sternum with tweezers and with scissors making a small hole into the diaphragm. A cut in both sides directed from this opening toward the forelimbs opens the thoracic wall (Figures 2.25a and 2.25b) and moves it in the cranial direction (Figure 2.26).

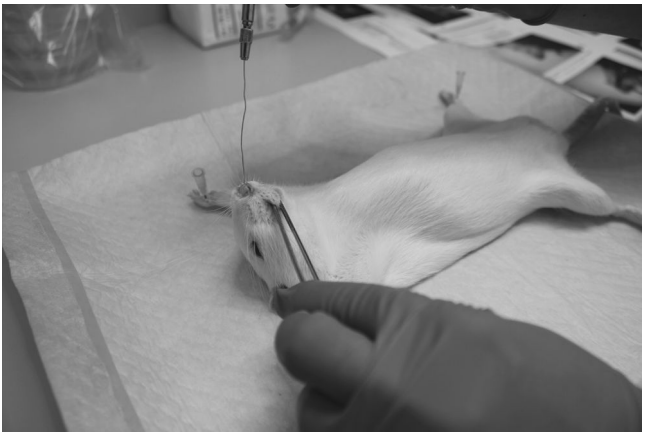


Figure 2.12 The platinum needle is introduced deeply into both sides of the nasal cavity.

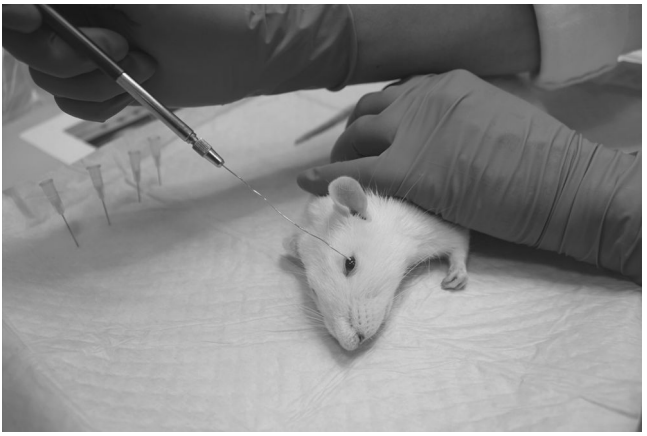


Figure 2.13 The conjunctiva is touched with the needle.

2.3.7 Sampling from diseased animals

Examination of diseased animals is a simpler matter than examination of healthy animals. The disease symptoms usually derive from certain organs, an abscess, or the equivalent, which can be sampled directly. Often, the decision to carry out bacteriology is made during a necropsy procedure. It is therefore essential for the bacteriological examination that



Figure 2.14 Sampling from the middle ear is facilitated by removing the entire auricle.

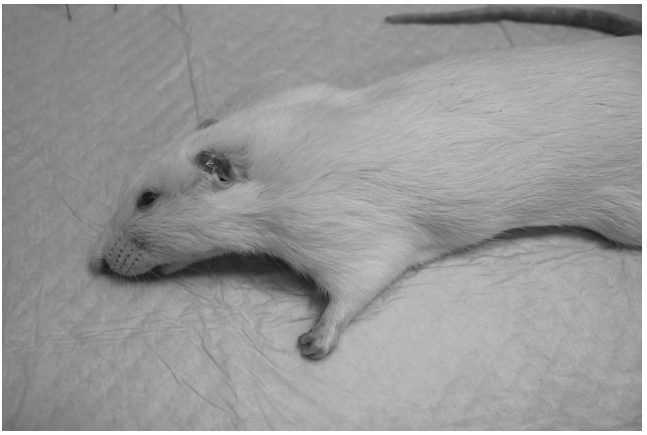


Figure 2.15 After removing the auricle, the membrane is accessible and may be opened with a scalpel.

sterility is maintained during such a necropsy procedure until it can be decided whether bacterial cultivation will be performed.

When sampling from diseased organs, the causative agent will often be found as a pure culture and as such will be much easier to handle. Alternatively, if a pure culture is not found, it can often be determined that the bacterial infection in the organ is secondary to a nonbacterial etiology.

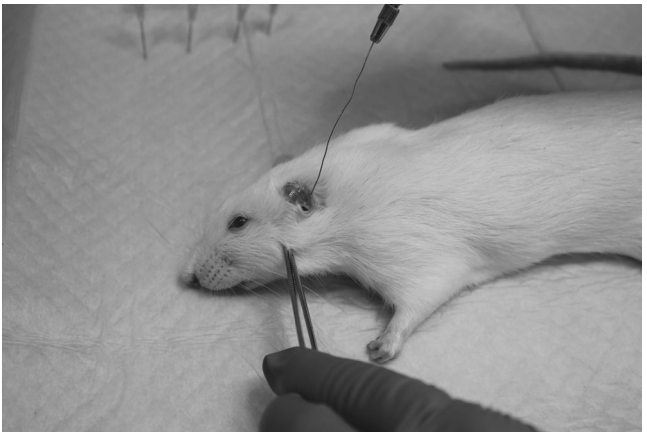


Figure 2.16 Sampling from the middle ear with a platinum needle.



Figure 2.17 The skin in the ventral neck region is opened to access the trachea for sampling, and the muscles over the trachea are bluntly spread.

2.3.8 Sampling from live animals

In some studies, it is necessary to sample from the gut or respiratory system of animals bound for survival postsampling.

2.3.8.1 Sampling from the gut

The most applicable way to obtain a sample of the gut microbiota of a live rodent is to remove the tip of the cecum surgically.[42] Instruments must



Figure 2.18 The trachea is lifted by placing the scissors underneath it.



Figure 2.19 The trachea is opened with a longitudinal cut.

be handled aseptically and the animal draped as for any surgical procedure, details of which may be found elsewhere.[43] The animal is anesthetized as described in Section 2.3.3. An approximately 1.5-cm incision (mouse) is made with a scalpel in the ventral abdominal middle line, and the muscle layer is separated from the skin layer and cut open using scissors. The cecum is lifted out through the wound after locating it with a tissue forceps, and a suture is made, leaving approximately 75% of the cecum on the dead-end side. The tip is removed using scissors and put



Figure 2.20 The platinum needle is introduced into the trachea in both the cranial and the caudal direction.



Figure 2.21 The skin is cut to access the abdomen.

in a sterile Eppendorf tube. The small amount of tissue left between the suture and the cut is cauterized, and some drops of an antibiotic solution (Tribrissen® [400/80 mg/ml sulfadiazin/trimethoprim] diluted 1:5 with sterile water) is dripped on the suture before the cecum is put back inside the mouse body. The muscle and then the skin layers are carefully sutured using sterile absorbable suture. After the procedure, the mouse is injected subcutaneously with 0.3 ml of saline water and some analgesic (e.g., 0.04 ml of Rimadyl® [50 mg/ml Carprofen] diluted to a concentration of 2.5 mg/ml). Postsurgically, animals must be treated with antibiotics,

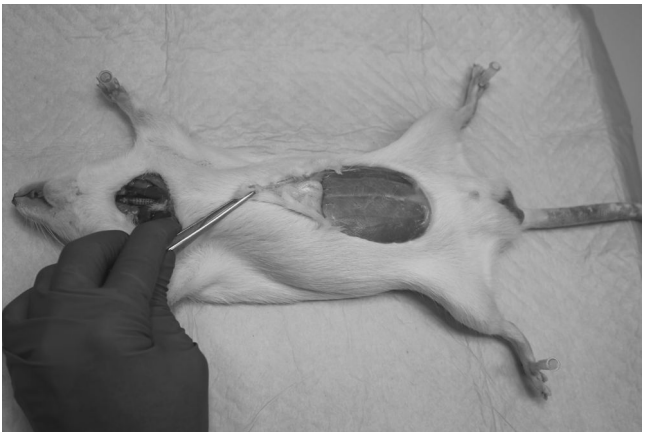


Figure 2.22 The abdominal skin is removed in the longitudinal direction of the animal all the way from above the pecten ossis pubis to the sternal manubrium.



Figure 2.23 A piece of the abdominal wall with muscles and peritoneum is removed from the pecten ossis pubis to the manubrium to access the abdomen.

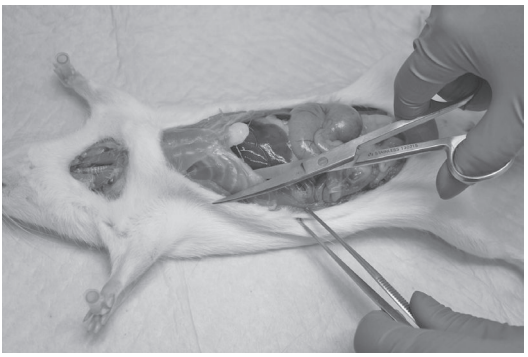
such as Ampivet® (250 mg/ml ampicillin), using 1.5 ml in 300 ml of water for 3–5 days. The microbiota will normalize approximately 1 week after antibiotic treatment.[42]

2.3.8.2 Sampling from the respiratory pathways

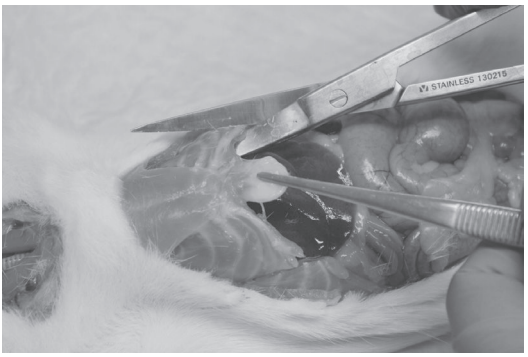
It is possible to obtain access to the respiratory pathways through two routes. It is possible to anesthetize rats and mice as described in Section 2.3.3 and intubate them (e.g., using the Hallowell Intubation Packs



Figure 2.24 After removing the abdominal skin, muscles, and peritoneum, the abdominal organs can be located and sampled.



(A)



(B)

Figure 2.25 (A) and (B) After removing the ventral skin, the thorax is opened by lifting the manubrium sternum with tweezers and making a small hole into the diaphragm with scissors. The thoracic wall is opened with a cut in both sides directed from this opening toward the forelimbs.



Figure 2.26 The thoracic wall is removed in the cranial direction to access the thorax for sampling.

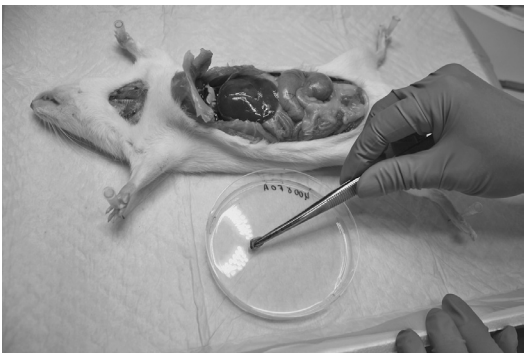


Figure 2.27 The cut surface of the lung piece is pressed onto the surface of an agar plate.

and workstations).[44] Obviously, as one has to pass through the mouth into the trachea, it is not only the deeper pathways that are sampled. Another option therefore is to shave or pluck the animal on the ventral neck region while the animal is placed on its back. The skin is cut a few millimeters in the longitudinal direction, and the muscles covering the trachea are bluntly dissected to the lateral sides to gain access to the tracheal surface. A 24-gauge catheter with a stylet (e.g., a Neoflon®) is inserted between the tracheal rings in the caudal direction and may be inserted as far as possible without using force. It is possible, at least in rats, to use lavage (0.1 ml),[45] but the safest method is to withdraw the catheter and simply cut off its tip into a sterile medium.



Figure 2.28 A piece of the cecal wall (major curve, upper side, midsection) is cut off with the scissors to access the cecal contents for sampling.

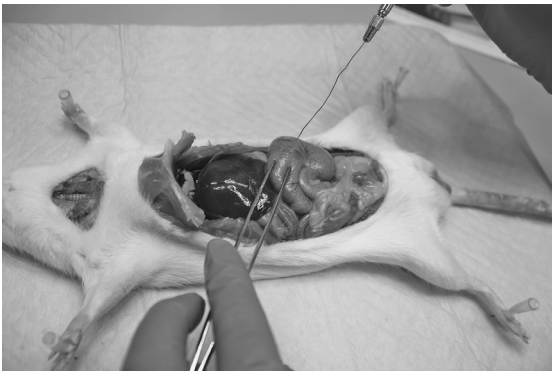


Figure 2.29 Sampling from the cecal contents.

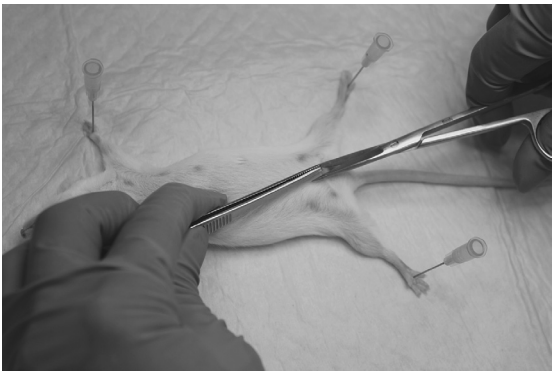


Figure 2.30 To access the female genitals for sampling, the vulvae are cut off with the scissors.

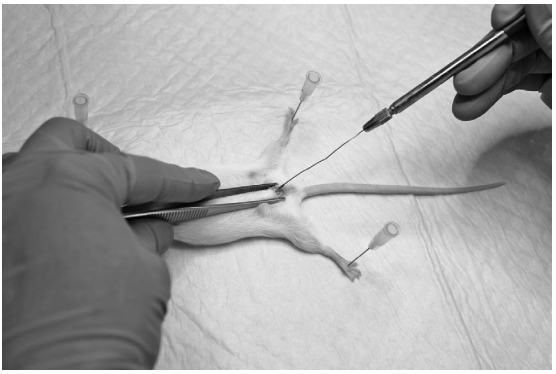


Figure 2.31 The platinum needle is introduced deeply into the vagina after the vulvae have been removed.

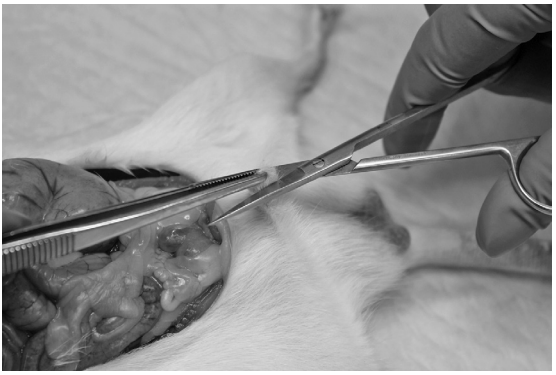


Figure 2.32 To access the male genitals for sampling, the preputium is cut off.



Figure 2.33 The glans is touched with the platinum needle after the preputium has been removed.

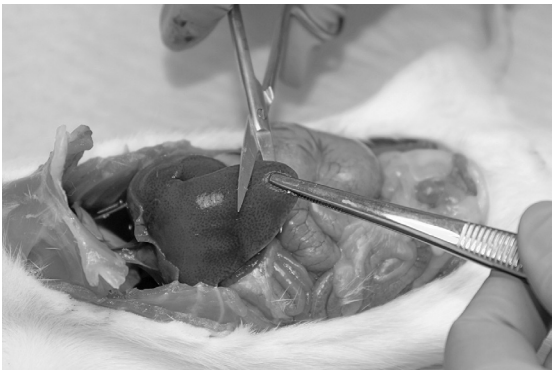


Figure 2.34 A lobe of the liver is grasped with the tweezers, and the grasped piece is cut off.



Figure 2.35 The cut surface of a piece of the liver is streaked on an agar plate.

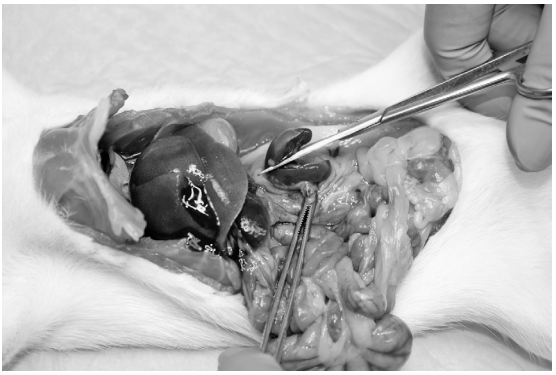


Figure 2.36 The kidney is halved in the pole-to-pole direction to access the renal pelvis for sampling.

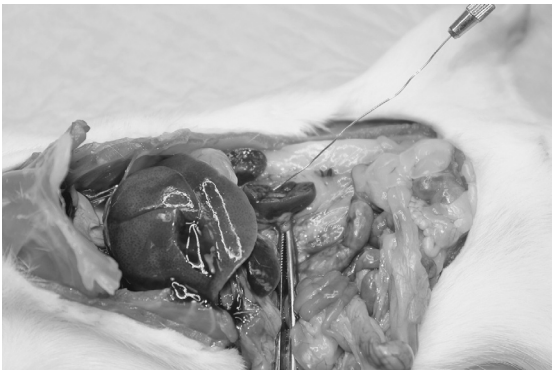


Figure 2.37 The surface of the renal pelvis is touched with the platinum needle.

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chapter three

Cultivation and identification of bacteria

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3.1 The basic steps in cultivation

In routine health monitoring with a broad scope of diagnoses, a range of selective and nonselective media may be applied. The most applicable way is to have selective media for all bacterial species to be searched for, but, for example, for *Pasteurella* spp., this is difficult. Therefore, nonselective media have to be applied, which subsequently leads to some work

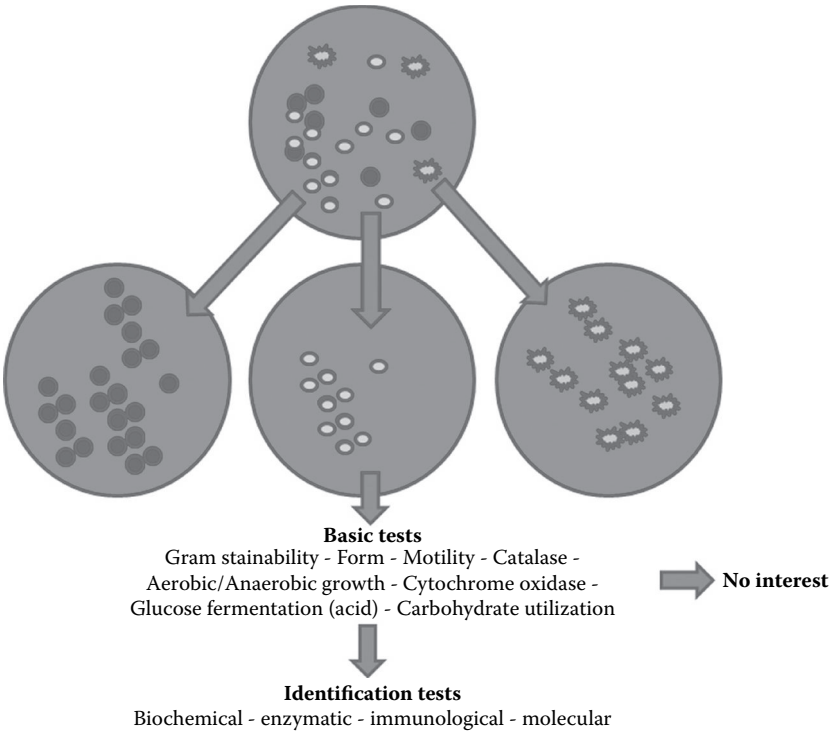


Figure 3.1 The various steps in cultivation and identification of bacteria from laboratory animals. Agar plates are inoculated from a certain organ, which leads to a mixture of several types of bacteria (primary culture), which can be distinguished by their colony morphology. A representative of each type of colony is picked and grown as a pure culture. Each pure culture is subjected to a range of basic tests. If the basic tests allocate the bacteria to groups not containing any of those bacterial species searched for, no further work is done on that culture. If, however, the bacteria are allocated to groups containing bacterial species of interest, a range of tests is performed until isolates have been identified.

identifying and isolating the various species, alternatively excluding their presence in the animals. This process of screening for various bacteria is shown in Figure 3.1.

3.2 The choice of media

Most screening programs consist of a combination of selective and nonselective methods. Also, when cultivating from pathologically changed organs, nonselective medium may be necessary for diagnosing nonspecific bacterial infections, which are often secondary to some other cause of disease.

The most common nonselective medium is 5% blood agar (see Table C.1). This medium will support the growth of both bacteria with low nutrient demands (e.g., Enterobacteriaceae or Micrococcaceae) and bacteria that will only grow in enriched media (e.g., Streptococcaceae). An advantage of this medium is that hemolysis is directly observable on the primary culture. However, this medium may be insufficient for some groups of bacteria, such as Pasteurellaceae, which by some is considered an important family in laboratory animal bacteriology. For example, primary growth of *Pasteurella pneumotropica* is only supported by some types of blood, a phenomenon that is not clearly understood; that is, it is difficult to give guidance for which types of blood should be used. It is therefore necessary to test blood from different sources in each laboratory before the method is reliable. Furthermore, blood agar does not support the growth of *Haemophilus* spp. Instead, chocolate agar (see Table C.6) is likely to support growth of most Pasteurellaceae. This agar is also suitable for anaerobic cultivation if 0.001 g of vitamin K and 0.55 g of cysteine HCL per 1000 ml are added to support growth of *Bacteroides* spp.

A main problem when using nonselective media is swarming of *Proteus* spp., a phenomenon that may totally inhibit the isolation of anything else. This is especially observed after cultivation from the cecum and the genitals. This is avoided by the addition of detergents, antibiotics, antisera, or anesthetics to the medium. Detergents and anesthetics are preferable as they do not hinder the isolation of *Proteus* itself, only the swarming. Dodecylebenzole sulfonate (0.005%) or chloral hydrate (0.1%) is usable.

For many bacteria, efficient selective, indicative, or combined selective-indicative media are optimal. Typically, this includes primary inoculation of a selective enrichment broth for propagation followed by the subsequent streaking of an indicative agar. This is especially usable for bacteria only found in low numbers in each animal. Some examples of such media are described in Appendix C of this book.

3.3 Incubation of media

Nonselective media are generally incubated at 37°C. Depending on which bacteria are included in a screening program, incubation may be aerobic, microaerophilic, or anaerobic. For aerobic incubation, the media are simply placed in the incubator. Microaerophilic incubation may be achieved either in a carbon dioxide incubator or by certain commercial systems for microaerophilic incubation, such as the Anaerocult C® or Anaerocult A® from Merck Millipore, Germany. Such systems usually consist of a package with a chemical that can be activated by the addition of water. The agar plates and the microaerophilic generation system are placed together in either a sealed plastic pack, such as the Anaerocult A™ (Merck Millipore)

or a closed jar, such as the Anaerocult C™ (Merck Millipore). An indicator stick, also available from the same commercial suppliers, should be placed in the jar or plastic pack. A simple reagent for anaerobic incubation may be produced on site in the laboratory (see Table C.5).

Aerobic or microaerophilic nonselective media are normally incubated for 18 to 24 h, inspected, reincubated for another 18 to 24 h, reinspected, and reincubated for the final 18 to 24 h, inspected, and discarded. Anaerobic media are generally incubated for 72 h before opening the incubation system.

Selective or indicative systems for specific bacteria should be incubated as described in the more specific chapters of this book.

3.4 Isolation of bacteria

Only pure, noncontaminated cultures should be subjected to identification. To make a pure culture from every single colony from the primary plates would be virtually impossible. Therefore, a representative of each morphologically distinct type on the plate is picked up. Often, to make the work more rational, plates from different organs, different animals, or both are grouped, and only one representative of each morphologically distinct colony type within each group is picked up and grown as a pure culture. To do this in a representative way, the group within which colonies are compared must be clearly defined (Table 3.1), for example, as either within the same animal but from different organs or within the same organs but from different animals.

Colonies are picked from indicative media in the same way, but only colonies with characteristics like those searched for are picked. To facilitate this process, it is a great help to grow a plate with a reference strain for each of the organisms to be isolated.

3.5 Initial characterization of the isolates

When pure cultures are obtained on nonselective agar plates, each culture should be subjected to some basic tests (Figure 3.1). First, the Gram stainability and shape of the bacteria have to be characterized as either Gram positive or Gram negative and either coccus or rod, respectively (Figure 3.2). Other basic characteristics are motility, aerobic and anaerobic growth, catalase, cytochrome oxidase, glucose fermentation (acid formation), and whether carbohydrates are utilized fermentatively or oxidatively. For Gram-positive bacteria, acid stability and the formation of spores are basic characteristics as well. When these basic characteristics have been verified, every isolate should be allocated to one of the groups shown in Tables 3.2 and 3.3, and a full identification may be carried out according to the specific chapters of this book.

Table 3.1 Isolation of Bacteria from Primary Plates

Organ	Animal number									
	1	2	3	4	5	6	7	8	9	10
A. Individual animal/individual organ isolation										
Nose	**1, 2, 3, 4**	**5, 6, 7**	**8, 9, 10 11**	**12, 13**	**14, 15 16**	**17**	**18, 19**	**20, 21, 22, 23, 24, 25**	**26, 27, 28**	**29, 30**
Trachea	**31**		**32**	**33, 34**	**35, 36**	**37, 38, 39**	**40**			**41**
Cecum	**42, 43, 44, 45**	**46, 47, 48**	**49, 50, 51, 52**	**53, 54, 55, 56, 57**	**58, 59, 60, 61**	**62, 63, 64, 65, 66, 67**	**68, 69, 70**	**71, 72, 73, 74, 75**	**76, 77, 78, 79, 80**	**81, 82, 83, 84**
Genitalia	**85, 86**	**87, 88, 89**	**90, 91, 92**	**93, 94, 95, 96**	**97, 98**	**99, 100**	**101, 102, 103**	**104, 105, 106, 107, 108**	**109, 110, 111, 112, 113**	**114, 115, 116, 117, 118**
B. Individual animal/grouped organ isolation										
Nose	**1, 2, 3, 4**	**9, 10, 11**	**17, 18, 19, 20**	**27, 28**	**36, 37, 38**	**44**	**56, 57**	**66, 67, 68, 69, 70, 71**	**80, 81, 82**	**92, 93**
Trachea	2		18	27, **29**	36, 38	**45, 46, 47**	**58**			**94**
Cecum	**5, 6, 7, 8**	**12, 13, 14**	**21, 22 23, 24**	**30, 31, 32, 33, 34**	**39, 40, 41, 42**	**48, 49, 50, 51, 52, 53**	**59, 60, 61**	72, 73, 74, 75, 76	**83, 84, 85, 86, 87**	**95, 96, 97, 98**
Genitalia	1,7	13, **15, 16**	17, **25, 26**	31, 33, 34, **35**	41, **43**	**54, 55**	56, 60, **62**	66, 75, **77, 78, 79**	86, **88, 89, 90, 91**	96, **99, 100, 101, 102**
C. Grouped animal/individual organ isolation										
Nose	**1, 2, 3, 4**	2, 4, **5**	1, 2, 5, **6**	12, 13	2, 4, 5	1	1, 3	1, 3, 4, 5, **7, 8**	3, 4, 8	2, 6
Trachea	**9**		9	9, **10**	9, 10	9, 10, **11**	**12**			10

continued

Table 3.1 (continued) Isolation of Bacteria from Primary Plates

	Animal number									
Organ	1	2	3	4	5	6	7	8	9	10
Cecum	**13, 14, 15, 16**	13, 14, 15	13, 14, 15, 16	13, 14, 15, 16, **17**	13, 14, 16, 17	13, 14, 15, 16, 17, **18**	15, 17, 18	14, 15, 16, 17, 18	14, 15, 16, 17, 18	15, 17, 18, **19**
Genitalia	**20, 21**	**20, 21, 22**	20, 21, 22	20, 22, **23, 24**	23, 24	**25, 26**	20, 22, 23	20, 21, 22, 24, 26	20, 21, 22, 24, 26	20, 21, 22, 23, **27**
	D. Grouped animal/grouped organ isolation									
Nose	**1, 2, 3, 4**	2, 4, **5**	1, 2, 5, **6**	2, 4	2, 4, 5	1	1, 3	1, 3, 4, 5, **7, 8**	3, 4, 8	2, 6
Trachea	2		2	2, 5	2, 5	2, 5, **6**	7			5
Cecum	**8, 9, 10, 11**	8, 9, 10	8, 9, 10, 11	8, 9, 10, 11, **12**	8, 9, 11, 12	8, 9, 10, 11, 12, **13**	10, 12, 13	9, 10, 11, 12, 13	9, 10, 11, 12, 13	10, 12, 13, **14**
Genitalia	1, **15**	1, 15, 12	1, 15, 12	1, 12, 11, **16**	11, 16	14, **17**	1, 12, 11	1, 15, 12, 16, 17	1, 15, 12, 16, 17	1, 15, 12, 11, **18**

Note: In this example ten animals were sampled, and inoculations were made from the nose, the trachea, the cecum, and the genitals from each animal on, for example, chocolate agar. After incubation for 18 to 24 h, each plate contains a number of morphologically distinct bacterial colonies. The four report sheets (A to D) in this table show four different systems for isolation of bacteria from the primary plates. Each number represents one specific type of colony, but the colony is only picked up from that plate in which it has been given in **bold** in the schedule; that is, the same number given at different locations in the schedule means that the two colonies have been regarded as morphologically alike. If the bacteria of each plate are only compared with bacteria on the same plate and not with any bacteria found on the other plates (i.e., each organ of each animal is examined independently of all other organs whether from the same or from the other animals) (A), 118 pure cultures have to be made, and afterward each must be passed through an identification process. This is a safe system but also is extremely expensive and time consuming. If, instead, the agar plates of the same animal are compared with one another, the number of isolates can be reduced to 102 (B). Alternatively, the individual animals may be kept separate, and the agar plates from the same organs may be compared with one another, which in this example reduces the number of isolates to 27 (C). The lowest number of isolates (i.e., 18) comes from comparing all plates with one another, regardless of the origin (D). The investigator should make clear which system is used, and although only individual animal/individual organ isolation (A) gives exact data, it should be stated in the examination report which bacteria were found in which animals. In general, grouped animals/individual organ isolation (C) is fully usable for routine examinations.

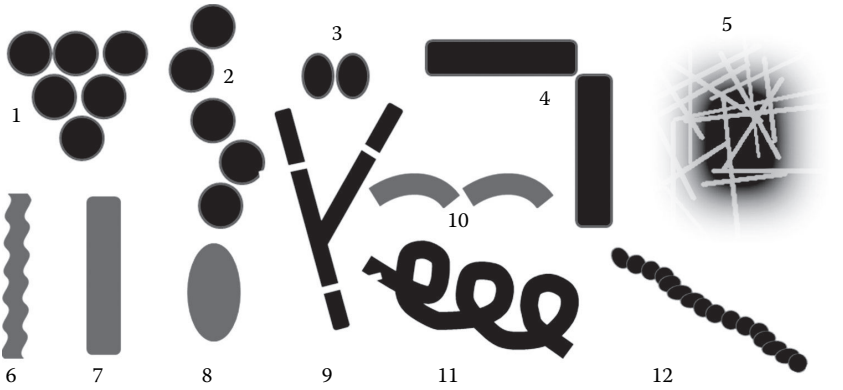


Figure 3.2 (See also color figure in the insert) Different shapes of bacteria found at microscopy. Items 1 and 2 are typical Gram-positive cocci (1) grouped as grapes and (2) grouped in chains; (3) if Gram-positive shapes, they also are designated as cocci, although they are more elliptic; as these are grouped as pairs, they are often designated diplococci. Items 4 are coryneform; 5, long, slender rods lying in bundles; 6, helical rod. Items 7 and 8 are typical for Gram-negative rods: Item 7 is a coliform, and item 8 is a pasteurellaform. Item 9 is a branching, filamentous rod; 10, curved rods in a pair, appearing as a seagull. Item 11 forms tight coils or spiral configurations, and item 12 is segmented and filamentous. 1–4, 9, 11, and 12 stain Gram positively (i.e., blue), 6–8 and 10 stain Gram negatively, and 5 is shown with immunofluorescence staining. Laboratory animal bacteria representing the different forms are 1, *Staphylococcus aureus*; 2, *Streptococcus zooepidemicus*; 3, *S. pneumoniae*; 4, *Corynebacterium kutscheri*; 5, *Clostridium piliforme*; 6, *Helicobacter hepaticus*; 7, *Escherichia coli*; 8, *Pasteurella multocida*; 9, *Actinomyces* spp.; 10, *Campylobacter coli*; 11, *Clostridium spiroforme*; 12, *Segmented filamentous bacteria* (*Candidatus savagella*).

In a particular program for bacteriological screening of laboratory animals, which bacteria to search for should be defined, for example, as shown in Table 3.4. After carrying out the basic tests on all isolates, some isolates may have been allocated to groups that do not contain bacteria listed in the program. In specific screening programs, these isolates may be disregarded without any further examination.

3.6 Conclusive identification

This book provides information that should help with the identification of important laboratory animal bacteria. Other bacteria found by cultivation may be identified with the help of international indexes for bacteria, such as *Cowan and Steel's Manual for the Identification of Medical Bacteria*[1] and *Bergey's Manual of Systematic Bacteriology.*[2] It should be kept in mind that many isolates possess characteristics that will place them between two or more well-defined bacteria; therefore, as such they cannot be fully defined without the use of methods based on molecular biology.

Table 3.2 First-Stage Table for the Identification of Gram-Positive Bacteria Found in Rodents and Rabbits

	Acid fast	Spores	Motility	Aerobic growth	Anaerobic growth	Catalase	Cytochrome oxidase	Glucose utilization	Carbohydrate utilization	Chapter of this book to be used for further identification
Cocci										
Micrococcus	–	–	–	+	–	+	–	d	O/–	12
Staphylococcus	–	–	–	+	+	+	–	+	F	8
Streptococcus	–	–	d	+	+	–	–	+	F	8
Rods										
Actinomyces	–	–	–	–	+	–	?	d	F	8
Arcanobacterium	–	–	–	+	+	–	–	+	F	8
Bacillus	–	+	d	+	d	+	d	d	F/O/–	8
Clostridium	–	+	d	–	+	–	?	d	F/–	8
Corynebacterium	–	–	–	+	+	+	–	d	–/F	8
Erysipelothrix/ Lactobacillus	–	–	–	+	+	–	–	+	F	8
Listeria	–	–	+ (20°C)	+	+	+	–	+	F	8
Mycobacterium	+	–	–	+	d	+	–	+	O/–	12

Table 3.3 First-Stage Table for the Identification of Gram-Negative Bacteria Found in Rodents and Rabbits

	Motility	Aerobic growth	Anaerobic growth	Catalase	Cytochrome oxidase	Glucose (acid)	Carbohydrate utilization	Chapter of this book to be used for further identification
Facultative anaerobes								
Enterobacteriaceae	d	+	+	+	–	+	F	10
Pasteurellaceae	–	+	+	+	+[a]	+	F[b]	10
Streptobacillus moniliformis	–	+	+	–	–	+	F	13
Obligate aerobes								
Bordetella	+	+	–	+	+	–	–	10
Pseudomonas	+	+	–	+	+	+	O	10
Microaerophilics								
Campylobacter/ Helicobacter	+	–[c]	+	+	+	–	–	10

[a] Dependent on the method applied.
[b] *Haemophilus* spp. do not grow in Hugh and Leifson's medium.
[c] Grows at microaerophilic incubation.
[d] 11–89% positive.

Table 3.4 Example of a Program for Routine Bacteriological Examination of Laboratory Rodents and Rabbits

Organ	Medium	Incubation	Bacteria screened for	
Nose	Chocolate agar	Microaerophilic, 37°C, 24 h	*Bordetella bronchiseptica*, *Haemophilus* spp., *Klebsiella pneumoniae*, *Pasteurella* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumoniae*	
	20% serum agar	Microaerophilic, 37°C, 3 days	*Streptobacillus moniliformis*	
Trachea	Chocolate agar	Microaerophilic, 37°C, 24 h	*Bordetella bronchiseptica*, *Haemophilus* spp., *Klebsiella pneumoniae*, *Pasteurella* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumonia*	
			Pasteurella spp.	PCR
	20% serum agar	Microaerophilic, 37°C, 3 days	*Streptobacillus moniliformis*	
	Blood agar with chloral hydrate	Aerobic, 37°C, 24 h	*Citrobacter rodentium* (mice only), *Klebsiella pneumoniae*, *Pasteurella* spp., *Salmonella* spp., *Yersinia* spp.	
	Selenite broth	Aerobic, 37°C, 24 h followed by streaking on BPLS agar	*Salmonella* spp., *Yersinia* spp.	
			Helicobacter spp.	PCR
	TVP agar	Microaerophilic, 37°C, 5 days	*Campylobacter* spp., *Helicobacter* spp.	

Table 3.4 (continued) Example of a Program for Routine Bacteriological Examination of Laboratory Rodents and Rabbits

Organ	Medium	Incubation	Bacteria screened for	
Genitals	Chocolate agar	Microaerophilic, 37°C, 24 h	*Bordetella bronchiseptica*, *Citrobacter rodentium* (mice only), *Haemophilus* spp., *Klebsiella pneumoniae*, *Pasteurella* spp., *Streptobacillus moniliformis*, β-hemolytic streptococci, *Streptococcus pneumonia*	
			Pasteurella spp.	PCR
Serum (antibodies)			CAR bacillus (rats only)	ELISA
			Clostridium piliforme	ELISA
			Mycoplasma pulmonis	ELISA

Note: BPLS agar = Brilliant-green Phenol-red Lactose agar; TVP agar = Trimethoprim, Vancomycin, Polymyxin B agar.

3.6.1 Specific techniques used for identification of bacteria

3.6.1.1 Gram-stainability tests

Gram stainability can be determined by the traditional Gram staining (Table 3.5) or by the less-laborious potassium hydroxide assay: A drop of 3% potassium hydroxide is placed on the object glass and a rich amount of bacterial mass is mixed into it. Because of hydrolysis of the cell wall, the DNA of a Gram-negative bacterium will form a mucoid immersion, and long threads (3 to 5 mm) can be drawn from the solution with the platinum needle after 15 to 20 s (Figure 3.3). A Gram-positive bacterium will form an aqueous immersion, from which no threads can be drawn. Commercial strips (Bactident® Aminopeptidase, Merck Millipore) may be used for testing for aminopeptidase activity, an enzyme normally found only in Gram-negative bacteria. A strip dipped into an Eppendorf tube with a mixture of water and bacteria is incubated with the mixture in a 37°C water bath for 10 min. If the mixture turns yellow, the bacterium is Gram negative. If no color evolves, the mixture is reincubated for 20 min. If there is still no change in color, the bacterium is Gram positive. If there

Table 3.5 A Method for Gram-Staining Bacteria

Materials		
Object glass	Lugol's solution	Microscope
Platinum needle	96% ethanol	Flame
Crystal violet	Weak carbol fuchsin	Immersion oil

Preparation

A drop of water is placed on the object glass, and two to three colonies are mixed into the water and spread over the surface of the glass. The glass is air dried and flame fixed.

Method

1. Crystal violet is spread over the surface of the glass and left for 1 min.
2. The glass is washed with Lugol's solution.
3. Lugol's solution is spread over the surface of the glass and left for 1 min.
4. The glass is washed with ethanol until the blue color disappears, at least outside the area where the bacteria have been spread.
5. The glass is washed with water.
6. Carbol fuchsin is spread over the surface and left for 15 to 20 s.
7. The glass is washed with water. A piece of filter paper is pressed onto the surface, and the glass is left until fully dried.

Microscopy of the Gram-stained slides

A drop of oil is placed directly on the surface of the glass without using a cover glass. Microscopy is done with an oil lens at ×1000 magnification. The shape of the bacteria is characterized according to Figure 3.2. Blue bacteria are Gram positive (Figure 3.2); red bacteria are Gram negative (Figure 3.2).

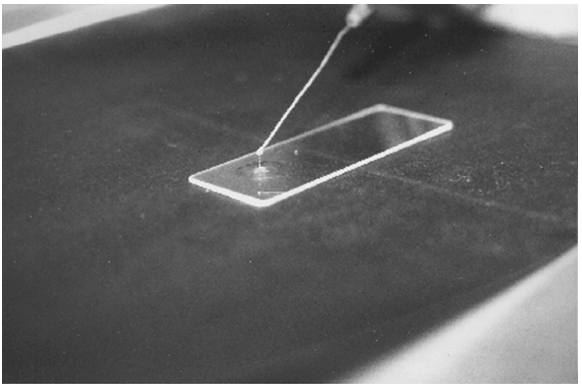


Figure 3.3 Drawing long threads from a Gram-negative bacterium after immersion into potassium hydroxide.

is only a weak change, the assay result is equivocal. As neither test is 100% reliable, it is often necessary to combine two or all three tests.

3.6.1.2 Other methods used for describing the shape of bacteria

Aqueous preparations may be directly microscoped. A drop of water is placed on the object glass, and two to three colonies are mixed into the water; a cover glass is placed on the mixture. Immersion oil is placed on the cover glass, and the preparation is microscoped at ×1000 magnification (oil lens). The bacteria are characterized according to Figure 3.2.

Alternatively, a colony may be mixed into India ink on an object glass. The mixture is spread in a thin layer and left until fully dry. The slide is microscoped as a Gram-stained slide. The microscoped field is dark, and the bacteria are seen as "holes" in the dark field.

3.6.1.3 Motility tests

Motility can be diagnosed by direct microscopy of a droplet from a broth. The bacteria should be moving in all directions—if they are moving in just one direction this is probably due to the moving of nonmotile bacteria by water streams on the slide. Alternatively, the bacteria may be inoculated in a semisolid medium (see Table C.2), which is inoculated and read as described in Figure 3.4.

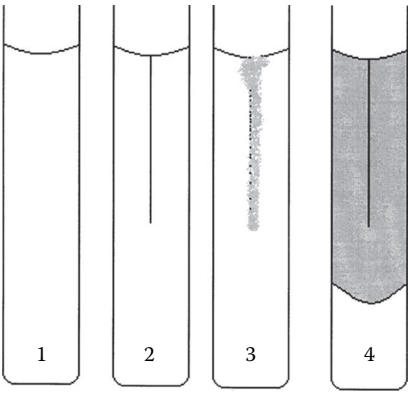


Figure 3.4 Inoculation of a semisolid medium for determination of motility of bacteria. The medium is only inoculated halfway from top to bottom (2). The medium is then incubated at 37°C and should be read after 6- to 8-h incubation and again after 18 to 24 h. If the bacterium is nonmotile, it will only grow along the inoculation channel (3); motile bacteria will spread from the channel in all directions (4).

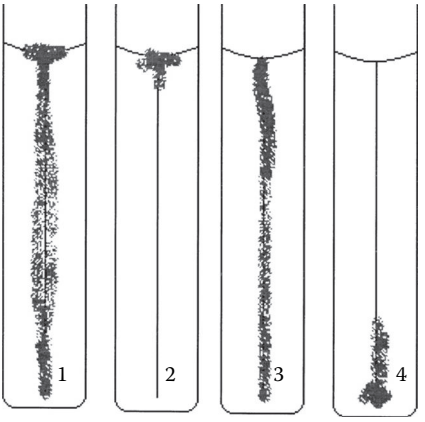


Figure 3.5 High-agar inoculation. A bacterial isolate has been inoculated from top to bottom in a tube and then has a 24-h incubation. 1, facultatively anaerobic; 2, obligate aerobic; 3, microaerophilic; 4, obligate anaerobic.

3.6.1.4 Test for aerobic and anaerobic growth

Testing for aerobic and anaerobic growth is done simply by incubating a pure culture on a nonselective medium under these conditions. Alternatively, a high agar (i.e., an appropriate medium in a high tube) may be inoculated and interpreted as described in Figure 3.5.

3.6.1.5 Catalase test

The test for catalase activity is performed by placing some colony mass on a slide and dripping 3% hydrogen peroxide onto the mass. If small bubbles develop, which may only be observed under a microscope, the test is positive. A commercial alternative is the Bactident™ Catalase reagents (Merck Millipore).

3.6.1.6 Cytochrome oxidase test

Testing for cytochrome oxidase activity is performed by dripping oxidase reagent (see Table C.3) onto some colony mass on a filter paper. A positive reaction is shown as a deep blue color within 10 s. It should be noted that this method, originally described by Kovács,[3] is more sensitive than a method later described by Gaby and Hadley.[4] Therefore, in some international indexes, *Haemophilus* spp. are described as oxidase positive, while in other indexes they are described as oxidase negative. A commercial alternative to this test is the Bactident® Oxidase strip (Merck Millipore), to which a colony can be added and the cytochrome oxidase reaction read within 1 min.

Table 3.6 A Method for Staining Acid-Fast Bacteria or Spores

Materials		
Object slides	Strong carbol fuchsin	1% malachite green
Burning flame	Ethanol or 5% sulfuric acid	Platinum needle

Preparation

A drop of water is placed on the object glass, and two to three colonies are mixed into the water, which then is spread over the surface of the glass. The glass is air dried and flame fixed.

Method

1. The carbol fuchsin solution is spread over the surface of the slide, which is then heated to boiling over the burning flame and left for 1 to 5 min.
2. The slide is washed under the water tap.
3. Spores
   The glass is washed with ethanol until the color disappears, at least outside the area where the bacteria have been spread.
   Acid-fast bacteria
   Sulfuric acid is used instead of ethanol.
4. The glass is washed with water.
5. Steps 3 to 4 are repeated until the smear is fully decolorized.
6. Malachite green is spread over the surface and left for 30 s.
7. The glass is washed with water. A piece of filter paper is pressed onto the surface, and the glass is left until fully dried.
8. The slide is microscoped as described for Gram-stained slides (Table 3.3).
   Spores
   Vegetative bacteria are green, and the spores are red. These may either be situated alone or be inside a bacterial cell. If so, they should be characterized as described in Figure 8.1.
   Acid-fast bacteria
   Acid-fast bacteria are red; other bacteria are green.

3.6.1.7 Acid-fast or spore staining

Acid-fast bacteria are resistant to destaining with sulfuric acid. A method for staining is shown in Table 3.6. This method may be modified to stain spores.

3.6.1.8 Carbohydrate fermentation and utilization assays

Basically, carbohydrate fermentation is tested in a broth supplemented with a specific carbohydrate and an indicator for acid production. A recipe is given in Table C.4. If a test for gas production is necessary, a so-called Durham tube, an inverted glass tube, is placed in the bottom of the broth. Whether the utilization of the carbohydrates is fermentative or oxidative should be tested in a medium described by Hugh and Leifson.[5]

3.6.1.9 Disk methods

Testing for sensitivity to antibiotics is done by agar diffusion inhibition assay, in which a disk with a minor amount of the antibiotic is placed on an agar plate with the isolate. After a cultivation period, an inhibition zone around the disk is measured. The concentration of the antibiotic decreases with increasing distance from the disk, dependent on the diffusion constant of the antibiotic. Therefore, the cutoff radius differs between different antibiotics. In practice, two cutoff radii are used, one below which the isolate is said to be *intermediate* resistant to the antibiotic, and a shorter radius, below which the isolate is defined as *resistant*. Radii higher than both cutoff radii indicate that the isolate is *sensitive.* The assay is performed on Mueller Hinton agar (see Table C.7). The antibiotic disks may be either prepared in the laboratory as paper disks soaked in an antibiotic solution or bought from commercial suppliers. They should be placed on the agar with an automatic dispenser. The isolate is suspended in 1 ml sterile distilled water to make a density of McFarland 0.5. The suspension is whirl mixed. A sterile cotton stick is dipped into the suspension several times, then surplus suspension is pressed out of the stick by pressing it against the inside of the tube. The cotton stick is then used for streaking on the agar surface, which is done three times, leaving no part of the surface untouched. Finally, the entire edge of the agar is touched by the stick all the way around. The agar is left to dry for 3 to 15 min, and the disks are placed with the dispenser. After 16 to 18 h of incubation at 37°C (staphylococci and enterococci should be incubated 24 h), the inhibition zones are read and interpreted according to the cutoff values given by the producer of the disks. As a rule of thumb, the cutoff values in Table 3.7 may be used.

3.6.1.10 Commercial test kits

Commercial kits containing a number of tests, which should lead to the full identification of bacteria belonging to the group for which the kit has been designed, represent an alternative to using a range of in-house tests. Some of these kits are for manual inoculation, reading, and interpretation, while others are partly or fully automated. One particular system, the API system sold by bioMérieux, France, has been widely used by laboratory animal bacteriologists because of its wide range of applications. It consists of a range of strips, each of which contains from 10 to 50 different substrates. Each strip is designed for a certain group of bacteria. The strips are inoculated according to given instructions, and then reactions are read; some tests demand the addition of a reagent prior to reading. Some kits are readable by an automatic reader. The results together lead to a numeric profile, which is computerized to provide the identity of the organism

Table 3.7 Presumptive Inhibition Zones for Testing Antibiotic Sensitivity by the Use of Antibiotic Disks on Mueller Hinton Agar[a]

	Diameter (mm) of inhibition zone	
	Sensitive	**Intermediate**
Ampicillin	19	16
Amoxicillin with clavulanic acid	19	16
Penicillin	25	25
Cephalosporins	27	23
Tetracycline	22	22
Chloramphenicol	25	22
Fucidine	27	23
Erythromycin	25	19
Tylosin	25	22
Spiramycin	25	22
Neomycin	22	19
Spectinomycin	19	16
Streptomycin	25	22
Lincomycin	25	22
Tiamuline	12	10
Sulfonamide	22	19
Trimethoprim	19	16
Sulfonamide with trimethoprim	25	22
Enrofloxacin	19	16
Gentamicin	22	19

[a] Neo Sensitabs®, Rosco Diagnostica (Denmark).

tested along with a probability of a correct diagnosis. The manual and the computer software base their identification on a percentage of standard results given by the manufacturer for each test and each bacterium (e.g., in the test for urease, 85% of *Pasteurella pneumotropica* isolates react positively). Commercial systems are extremely valuable for the laboratory animal bacteriologist. Table 3.8 lists the names of API strips suitable for important bacteria in laboratory animals. However, limitations on the use of such systems within laboratory animal bacteriology should be noted, and one should never rely solely on commercial kit identification. The following points are important:

1. The kits are designed for human use. Profiles of the bacteria included in the manuals or computer programs are not always directly comparable with animal strains of the same bacteria. For example, if rat

Table 3.8 API kits (bioMérieux, France) Suitable for the Diagnosis of Important Laboratory Animal Bacteria

	API kit
Bordetella bronchiseptica	20 NE
Citrobacter rodentium	20 E
Campylobacter spp.	Campy
Corynebacterium kutscheri	Coryne
Erysipelothrix rhusiopathiae	Campy
Haemophilus spp.	NH
Helicobacter spp.	ampy
Pasteurella spp.	20 NE
Salmonellae	20 E
β-Hemolytic streptococci	20 Strep
Streptococcus pneumoniae	20 Strep
Yersinia pseudotuberculosis	20 E

strains of a certain bacterium differ from human strains in a certain test, this usually will not be listed or will be listed only as a low-percentage reaction. Some bacteria (e.g., the murine *Citrobacter rodentium*) are not included in the API system; therefore, the profile must be known from other sources. API 20 NE may be excellent in characterization of Pasteurellaceae on the family level but unreliable on a genus level.[6]

2. The individual test results often may be used to make up a diagnosis without the use of the commercial manual or computer software. However, occasionally alternative indicators have been applied, which may lead to alternative results. If this is the case, a standard in-house test should be applied in the laboratory as a supplement.

To avoid false diagnoses as a result of using commercial kits, a diagnosis on a generic level should always be reached using basic standard tests before attempting full identification with a commercial kit. Furthermore, quality assurance of the kits should be performed by screening reference strains of laboratory animal bacteria in the kits used in the laboratory, and the profiles obtained by this procedure should be used along with manuals and computer software.

Some of the single assays to be performed exist as Bactident strips or reagents (e.g., in addition to those already listed, indole reagent is available).

3.7 Molecular biology-based methods for rapid identification of microorganisms

3.7.1 Extraction of DNA

Once a pure culture has been obtained, molecular biology offers an array of methods suitable for rapid and accurate identification of cultivable microorganisms. Most of these methods are DNA based, although RNA and protein-based methods also exist. For DNA-based methods, the first step after obtaining a pure culture is to extract DNA. This can be done either from broth or from a plate. Overall, the aim is to obtain DNA of sufficient quality and purity to allow subsequent downstream analysis. For some applications, mainly simple methods based on polymerase chain reaction (PCR), aiming at amplifying a specific, relatively short DNA fragment, rather simple methods such as boiling a loop full of colony mass in, for example, TE-buffer (10 mM Tris, 1 mM EDTA [ethylenediaminetetraacetic acid], pH 8.0) will work, but in general gentler methods are to be preferred. Numerous commercially available kits have been developed for the purpose. In all cases, it is essential to ensure efficient lysis of the cells. This is in general relatively straightforward for Gram-negative bacteria, but some Gram positives (many cocci, *Listeria*, some *Lactobacillus* and clostridia, etc.) can be difficult to lyse. Modification of the protocol supplied by the manufacturer by addition of a bead beating step (using, e.g., a FastPrep instrument, MP Biomedicals, Santa Ana, CA, USA); addition of additional enzymatic steps, such as addition of lysozyme; or incorporation of a boiling step might be necessary. For DNA–DNA hybridization studies, extremely gentle extraction of DNA is necessary.[7–9]

3.7.2 Grouping by repetitive DNA element PCR

If working with many isolates it can be an advantage to group the isolates to species or subspecies level before continuing with other methods, such as rRNA gene sequencing. Repetitive DNA element PCR (abbreviated to rep-PCR) is a rapid and robust method for this purpose. Short, repetitive elements are spread over the genome of both pro- and eukaryotes. During PCR, the regions between these repetitive regions are amplified. Subsequently, the PCR products are separated by agarose gel electrophoresis, resulting in a species-, subspecies-, or in some cases even strain-specific pattern (given that DNA is extracted gently). Several different primer sets have been published for the purpose. The primer $(GTG)_5$ (5′GTGGTGGTGGTGGTG3′, works both as a forward and a reverse primer) in general shows reliable grouping at the species or subspecies

level for almost all bacteria, yeast, and some fungi. For higher discriminatory power, other primer sets, such as the BOX, ERIC, and REP primers, might be suited, depending on the target in question. Using software for gel comparison, such as Gel Compare and BioNumerics (both from Applied Maths, Sint–Martens–Latem, Belgium), these profiles can then be compared and clustered. Isolates clustering together/having the same profile are then assumed to belong to the same group (e.g., species level), and instead of continuing with the identification of all isolates, a few, representative isolates from each cluster are then selected for further analysis.[8,10–12] If type strains or already-identified reference strains are included in the analysis, rep-PCR can in many cases be used directly for identification when new isolates cluster with the type or reference strain. This can be a huge advantage in the routine laboratory if a database with rep-PCR profiles of known isolates is built up over the years, allowing rapid and cost-efficient identification.

3.7.3 Identification by ribosomal RNA gene sequencing

The ribosomal RNA (rRNA) genes have a rather unique primary structure: Conserved regions interchange with variable regions. The conserved regions are assumed to be identical between, for example, all prokaryotes, thus sharing the same sequence in these regions; the variable regions differ much more in sequence. This makes the conserved regions ideal for designing "universal primers" that can be used to amplify the "unknown" variable regions in between. For bacteria, the 16S rRNA gene is the gene of choice for identification to species level. The primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′, which hybridizes to the beginning of the 16S rRNA gene) and 1492R (5′-TAC CTT GTT ACG ACT T-3′) amplifies almost the entire 16S rRNA gene and is widely used, although it (as with all other universal primer sets) is not 100% universal.[13] For yeast and yeast-like organisms, either the 18S rRNA gene or the D1/D2 region of the 26S rRNA gene [primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′)] is normally targeted; also, the internal replaced spacer (ITS) region [ITS1–5.8S rDNA-ITS2; primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′)], which generally has higher discriminatory power, can be used.[14,15] The rRNA genes can be used for fungi as well, but often the discriminatory power is too low, and, for example, the calmodulin gene has to be sequenced instead.[16]

Following PCR, the amplicons are purified and sequenced by classic Sanger sequencing, either in-house or by a commercial facility. Unless throughput of the laboratory is high, the latter is normally the economically better choice. Following sequencing, the sequences are trimmed

and manually corrected if needed. This can be done using shareware tools such as Chromas (http://technelysium.com.au) or more advanced, but also more expensive, tools such CLC Workbench (http://www.clcbio.com/products/clc-main-workbench/). Subsequently, a database search is carried out, normally using the BLAST algorithm.[17] GenBank offers the largest (but uncurated) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). When the purpose is identification to species or subspecies level, curated databases like EzTaxon (prokaryotes) and EzFungi (fungi) often yield more accurate and useful answers (http://www.ezbiocloud.net/).

Occasionally, sequencing of the 16S rRNA gene does yield unambiguous identification, as this gene differs only a little within some groups of bacteria. For example, this is the case for some lactic acid bacteria, *Bacillus* spp., and members of the Enterobacteriaceae. In this case, other genes, such as *rpoB*, *gyrA*, or *gyrB* have to be amplified and sequenced.[18–20]

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chapter four

Immunological methods

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4.1 Antigens and antibodies

Immunological methods, that is, methods based on an antigen–antibody reaction, are used in two ways: *antigen detection*, in which specific antibodies directed against a certain agent are used to show the presence of this agent in a sample or to identify a pure culture as the agent; and *serology*, in which the animal, by the use of the agent or parts of it as antigen, is shown to possess antibodies against that agent.

Immunological identification may be applied to a sample in which the presence of a certain bacterium is suspected. This requires either a large amount of the agent in the sample or a high sensitivity of the method, although a lower sensitivity may be accepted if identification is applied to pure cultures. In setting up the method (e.g., when pretesting the antibodies), attention is normally paid to avoiding cross-reactions with closely

related bacteria, while bacteria with a more distant relationship would not be tested. One should, therefore, never try to identify a pure culture by an immunological method if the culture has not been subjected to basic tests, and these tests have allocated the isolate to the group including that bacterium against which the antibody is directed.

If antigen detection is used to diagnose an infection, it will often be sufficient to immunostain a smear prepared from the infected organ. Pathologists might have an interest in describing the position of the antigen inside the tissue; therefore, they often stain slides prepared as for histopathology, a technique that is not covered by this book.

Serology is the art of identifying the presence of antibodies in serum. For those bacteria for which cultivation methods are too laborious, time consuming, or even impossible (e.g., *Clostridium piliforme*), the demonstration of antibodies may be a more reasonable approach. The main pitfalls are the lack of sensitivity because of a pure antibody response to the infection, which is especially the case for gut bacteria toward which the immune system in early life will develop oral tolerance (i.e., a response based on the regulatory T cells and innate responses and therefore not necessarily leading to systemic antibody responses).[1] Also, low specificity because of cross-reactions may commonly be observed, as typical bacteria may contain more than 2000 antibody-inducing epitopes. Antibodies against *C. piliforme*, cilia-associated respiratory (CAR) bacillus, *Mycoplasma* spp., and *Leptospira* spp. are easily revealed by serology because they induce a high amount of serum immunoglobulin (Ig) G, but specificity problems do occur.

4.2 Agglutination

The simplest immunological method is to agglutinate the antigen by a serum containing specific antibodies. The presence of antibodies in the serum is shown by positive agglutination. Alternatively, if the antigen is the unknown factor, the positive agglutination indicates the identity of the antigen. The method may be performed in a tube, on a slide, or on a piece of black cardboard. The visualization of the agglutination on a slide may be enhanced if the known factor (i.e., either the bacterial antigen or the specific antibodies) is linked to polystyrene (latex) particles, which are generally 1 μ in size. The method is not very sensitive as a tool for diagnosing antibodies, and in laboratory animal bacteriology, it is mainly used for diagnosing antibodies to *Leptospira* spp.[2] For diagnosing the presence of certain bacteria in a nonpurified sample, this method is normally too insensitive to be of any practical use, but as these tests require a minimum of time and skill for performance, they are extremely valuable and commonly applied for quick identification of a pure culture. Many bacteria will react directly with the antibodies; the reaction of others may

be enhanced if the bacteria have been digested by an enzyme. Latex-linked antibodies against various bacteria (e.g., *Staphylococcus aureus* and Lancefield's groups of streptococci) are commercially available.

4.3 Immunofluorescence techniques

4.3.1 Diagnosing the presence of bacteria in a sample

The principles of immunofluorescence techniques are shown in Figure 4.1. A specific antibody, preferably a monoclonal antibody, is used for staining of bacteria. The antibody should be pretested in different dilutions. The antibody is subsequently used in a dilution that is considerably lower than the highest dilution giving fluorescence at pretesting. An example of a protocol for indirect immunofluorescence staining is shown in Table 4.1 (also see Figures 4.2 through 4.4 pertaining to this table). Samples to be tested can simply be smeared on a Teflon-coated slide (Figure 4.2) and fixed using –20°C acetone. Formalin should not be used as this may cover the antigens for the antibody. Immunofluorescence staining is, in general, more sensitive than peroxidase-antiperoxidase staining (see Section 4.4). In laboratory animal bacteriology, the method has been applied as a simple tool for staining of *Clostridium piliforme* (see Figure 3.2, item 5). Fluorescein-conjugated, species-specific anti-immunoglobulins are available from several commercial suppliers. The method may as well be applied

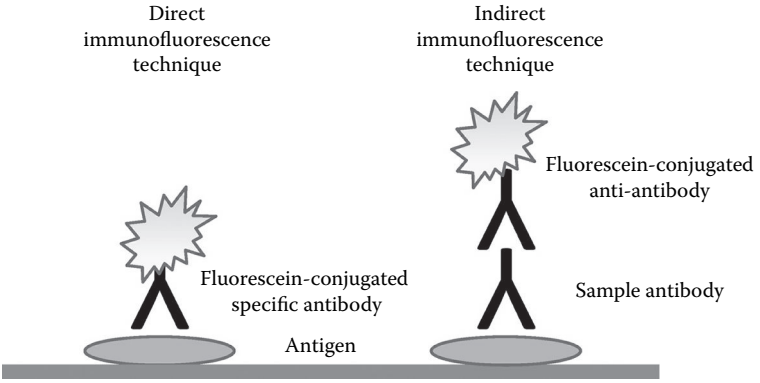


Figure 4.1 The principle of immunofluorescence techniques. The bacterial antigen is linked to a glass slide. If a specific fluorescein-labeled antibody is available, the bacteria are directly visualized. If such an antibody is not available or the possible detection of antibodies in a serum sample is the aim, the indirect technique is used: The specific antibody reacts with the bacterial antigen, and a fluorescein-labeled anti-antibody reacts with the specific antibody. In both techniques, subsequent microscoping in a fluorescence microscope will show fluorescent bacteria (Figure 3.2, item 5).

Table 4.1 Example of a Protocol for Immunofluorecence Staining for the Identification of Bacteria in Organ Samples

Materials

8- or 10-well Teflon slides
Arced tweezers
Acetone (–20°C)
Scissors
Filter paper
Phosphate-buffered saline (PBS)
Micropipette
Primary antiserum: Antiserum against the agent to be stained
Conjugate antiserum: Fluorescein-labeled antiserum against the immunoglobulin of the animal species delivering the primary antiserum
Humid chambers (Figure 4.2A)
Washing trunks (Figure 4.2B)
Cotton sticks
Filter paper
Fluorescence microscope (Figure 4.3)
Mounting fluid (e.g., 9:1 PBS glycerol)

Preparation

Tissue samples: A piece of the organ is sampled. With the arced tweezers, the cut surface is pressed gently against a piece of filter paper to remove excess blood, and a thin smear is made over the wells of the Teflon slides (cut surface toward the slide). The Teflon slides are left at room temperature until dry, then covered with –20°C acetone (*use a safety cabinet*), and again left until dry. If not stained immediately, the slides may be frozen at –20°C.

Pure cultures: A broth or a suspension is diluted until one drop contains several but not intermingling bacteria. One drop (10 to 15 µl) is placed in each Teflon slide well. The Teflon slides are left at room temperature until dry, then covered with –20°C acetone (*use a safety cabinet*), and again left until dry. If not stained immediately, the slides may be frozen at –20°C.

Staining

1. Dilute the primary antiserum and the conjugate antiserum in the proper dilution found at pretesting.
2. Place the slides in the humid chamber filled with water.
3. Put 12 µl diluted primary antiserum onto each well. Close the chamber and incubate at room temperature for 30 min.
4. Wash three times for 5 min each time in PBS in the washing trunks.
5. Dry all fluid between the wells by the use of the cotton sticks. Do not dry the wells.
6. Put 12 µl diluted conjugate antiserum onto each well. Close the chamber and incubate it at room temperature for 30 min.
7. Wash three times for 5 min each time in PBS in the washing trunks.
8. Leave the slides until dry.
9. Place a tiny drop of mounting fluid on each well. Avoid bubbles. Place a cover glass on the slide.
10. Perform fluorescence microscopy at ×400 or ×1000 (immersion oil) magnification.

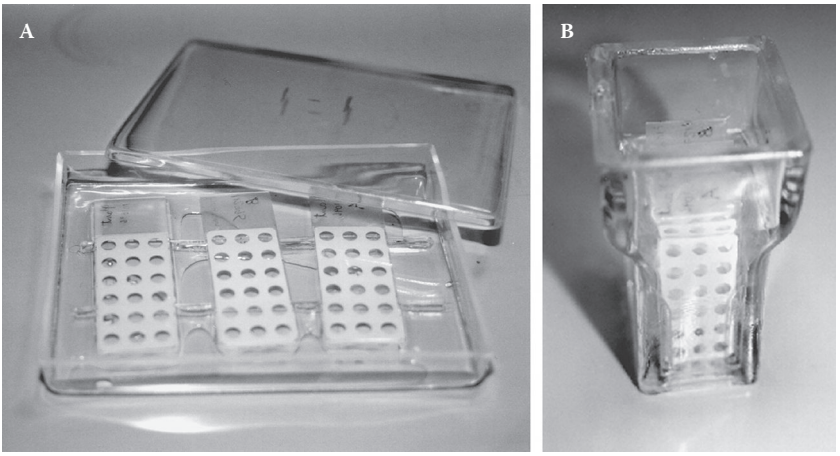


Figure 4.2 Teflon-coated slides for immunofluorescence staining placed in a humid chamber (A) and washing trunks for washing immunofluorescence slides (B).

on pure cultures for identification of these with specific antisera, as it may be done with mycoplasmas.

4.3.2 The immunofluorescence assay

Use of immunofluorescence techniques for demonstrating antibodies (serology) is in principle the same as demonstrating the antigen (see Figure 4.1). For demonstrating antibodies, only the indirect technique is usable, referred to as the *immunofluorescence assay* (*IFA*). This method is mostly applicable for diagnosing antibodies to bacteria of a certain size, as during microscopic evaluation it may be difficult to differentiate small bacteria from unspecific fluorescence. If the bacterium can be grown in superficial media, it is rather simple to inoculate a broth, incubate it, and dilute it until the bacterial density is acceptable for microscopy. A drop of the diluted broth is then put onto a Teflon-coated slide and fixed with –20°C acetone. For bacteria such as *C. piliforme*, which have to be harvested from experimental infections, a thin smear of the infected organ or cell culture has to be made as described in Table 4.1. For some agents, such as *C. piliforme* and CAR bacillus, antigen-coated slides are commercially available. In principle, the technique is performed in exactly the same way as described in Table 4.1 for staining slides. The test serum is diluted prior to testing. The safest way to make a diagnosis is to make serial dilutions of 1:20, 1:40, 1:80, up to 1:640 and then test all dilutions. A negative control serum from a verified uninfected colony should be included, and the

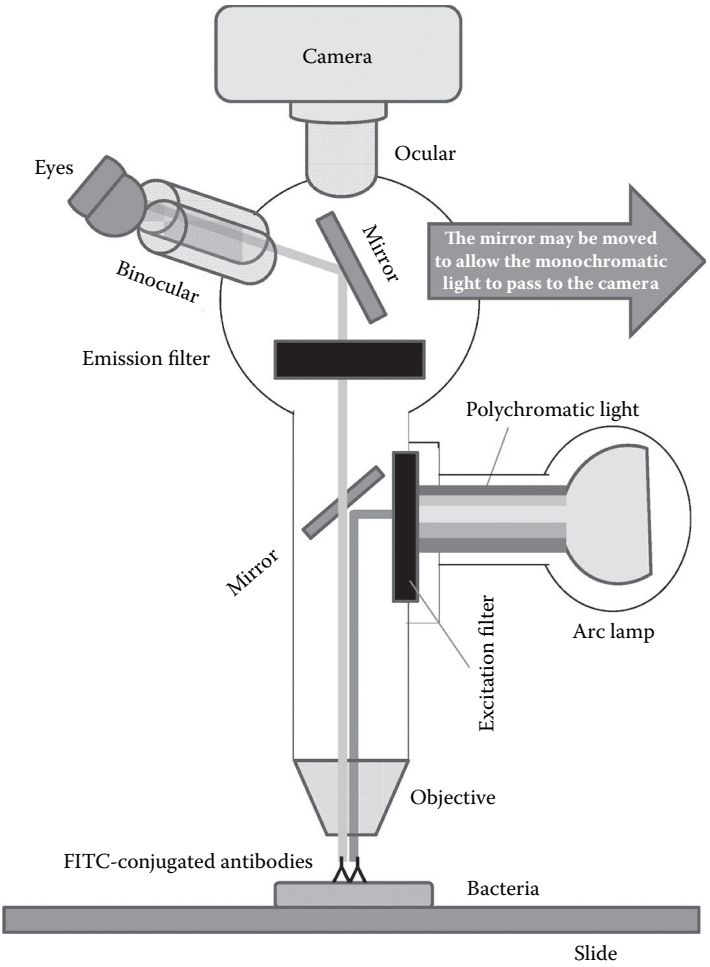


Figure 4.3 (See also color figure in the insert) Principles of a fluorescence microscope. An arc lamp generates a polychromatic light, which is filtered into monochromatic light. A mirror directs the light toward the specimen stained with conjugated antibodies, and light is returned in another wavelength capable of passing the emission filter. A mirror is used for directing the light into the binoculars but can be moved to let the light pass to the camera.

assay should be validated against several of such control sera to clarify at which dilution the control sera exhibit unspecific binding. However, various diagnostic laboratories often use only one dilution (e.g., 1:20 or 1:40) for testing, at which they validate the presence or absence of fluorescence, which is a more insecure but much cheaper diagnostic method.[3–5] If such a routine is practiced, at least positive samples should be rescreened by

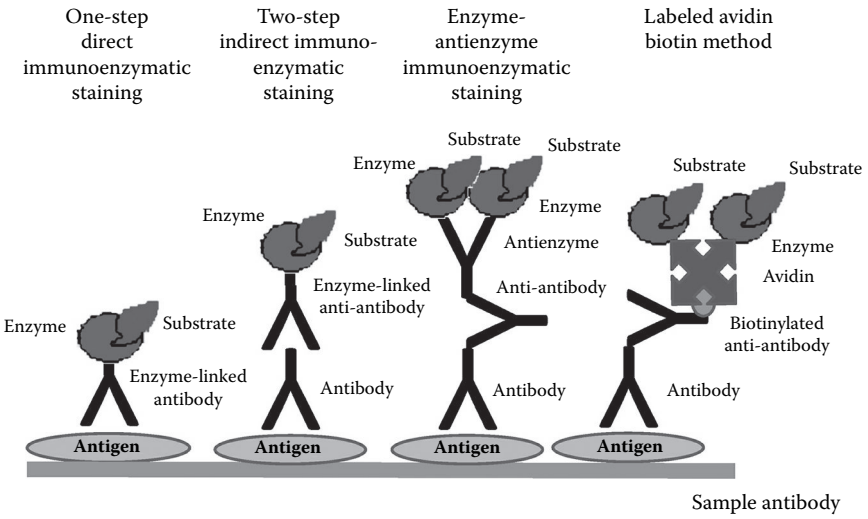


Figure 4.4 (See also color figure in the insert) Immunoenzymatic staining methods. The antigen may be stained directly using an enzyme-conjugated antibody specifically directed against the antigen. To raise the sensitivity, various indirect methods may be applied using antienzymes or the avidin-biotin system.

serial dilutions versus a negative control. Whatever method is applied, a positive control should always be included.

4.4 Immunoenzymatic staining

As an alternative to using fluorescein-conjugated antibodies for staining bacteria, antibodies conjugated with enzymes, such as peroxidase or alkaline phosphatase, may be used. The principle is shown in Figure 4.4. The slides can be microscoped in an ordinary light microscope, which facilitates better visualization of the in situ placement of the bacterium and makes immunological staining available to those lacking a fluorescence microscope. A clear disadvantage of the enzymatic technique is lower sensitivity, which, however, may be counteracted using the enzyme–antienzyme technique (Figure 4.4). This method is more sensitive because of the presence of more enzyme molecules. Staining with peroxidase, referred to as PAP (peroxidase-antiperoxidase) staining, is generally usable, but in some specimens, a high amount of endogenous peroxidase is present; under such circumstances, alkaline phosphatase should be used instead—so-called APAAP (alkaline phosphatase-antialkaline phosphatase) staining. A protocol for PAP staining is shown in Table 4.2. Another way of raising the sensitivity is to use the proteins avidin or streptavidin along with the coenzyme biotin. Avidin or streptavidin have a high affinity for biotin;

Table 4.2 A Suggested Protocol for Peroxidase-Antiperoxidase Staining

Materials

Washing bottle with distilled water
PBS
Washing trunk
Cotton sticks
Arced tweezers
Rabbit serum (dilution 1:20 in PBS)
Primary antibody (specific antibody to the agent in question, diluted 1:40 in PBS)
Humid chamber
Mounting fluid (e.g., 9:1 PBS glycerol)
Microscope
Anti-immunoglobulin (specific for the species delivering the primary antibody, diluted 1:40 in PBS)
Horseradish peroxidase-antiperoxidase (PAP) complex (antibody must be specific for the species delivering the primary antibody, diluted 1:40 in PBS)
DAB tablets
0.05M Tris buffer, pH 7.6
3% hydrogen peroxide

Preparation

A piece of the organ is sampled. With the arced tweezers, the cut surface is pressed gently against a piece of filter paper to remove excess blood, and a thin smear is made over the wells of the Teflon slides (cut surface toward the slide). The Teflon slides are left at room temperature until dry, then covered with –20°C acetone (*use a safety cabinet*), and again left until dry. If not stained immediately, the slides may be frozen at –20°C.

Preparation of the DAB substrate solution

Dissolve 6 mg DAB in 10 ml of the Tris buffer and add 0.1 ml hydrogen peroxide (should be filtered if precipitate forms). Solution is stable for 2 h at room temperature.

Method

1. Gently rinse the slide with distilled water from a wash bottle.
2. Place slide in PBS in the washing trunk.
3. Use a cotton stick for removing excess liquid from around the specimen.
4. Apply 4 to 6 drops of normal rabbit serum (dilution 1:20).
5. Tap off serum and wipe away excess.
6. Apply 4 to 6 drops of the primary antibody (dilution 1:40).
7. Incubate in a humid chamber for 30 min.
8. Repeat steps 1 to 5.
9. Apply 4 to 6 drops of anti-immunoglobulin (dilution 1:40).
10. Incubate in a humid chamber for 30 min.
11. Repeat steps 1 to 5.
12. Apply 4 to 6 drops of horseradish peroxidase-rabbit(-mouse)-antiperoxidase (PAP) complex (dilution 1:40).
13. Incubate in a humid chamber for 30 min.
14. Gently rinse the slide with distilled water from a wash bottle.
15. Use a cotton stick for removing excess liquid from around the specimen.

Table 4.2 (continued) A Suggested Protocol for Peroxidase-Antiperoxidase Staining

16. Apply DAB substrate solution to give a colored end product and incubate until desired color intensity has developed.
17. Gently rinse the slide with distilled water from a wash bottle.
18. Leave the slides until dry.
19. Place a tiny drop of mounting fluid on each well. Avoid bubbles. Place a cover glass on the slide.
20. Perform microscopy at ×400 or ×1000 (immersion oil) magnification.

that is, if a biotinylated antibody is used as the secondary anti-antibody, this may be visualized by the use of enzyme-labeled avidin molecules.

The substrates used for enzyme-linked immunosorbent assay (ELISA; see next section) should not be used for this technique because substrates should not be water soluble as they need to be for ELISA. For horseradish peroxidase, either 3′-diaminobenzidine tetrahydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC) is used; fast red or new fuchsin is used for alkaline phosphatase. All are commercially available.

Immunoenzymatic staining may be applied for staining a wide range of bacteria. Quick-staining kits only supplied with a specific antibody are commercially available.

4.5 Enzyme-linked immunosorbent assay

4.5.1 Principles

The principles of ELISA are shown in Figure 4.5. ELISA is usually performed in a 96-well microtiter plate. For diagnosing the presence of the antigen in a sample (Table 4.3) (e.g., *Salmonella* spp.), the capture technique is used; that is, the surface of the microwell is coated with an antibody. The application of ELISA for serology (Table 4.4) (i.e., demonstration of antibodies in serum) is one of the most widely used methods in health monitoring (e.g., as a tool for screening for *C. piliforme*[6] and *Mycoplasma* spp.[7]).

4.5.2 The microtiter plates

The microtiter plates are normally made of either polystyrene or polyvinyl chloride (PVC). The material is essential, and two types are important in bacterial serology: High-binding plates, also known as enhanced binding or maxisorp, bind approximately 400 ng protein/cm^2; and medium-binding plates, also known as polysorp, bind only approximately 100 ng protein/cm^2. High-binding plates will—if used with a potent blocker—be the first choice, but to avoid too high a background signal, it may be necessary to use medium-binding plates instead. To avoid the waste of

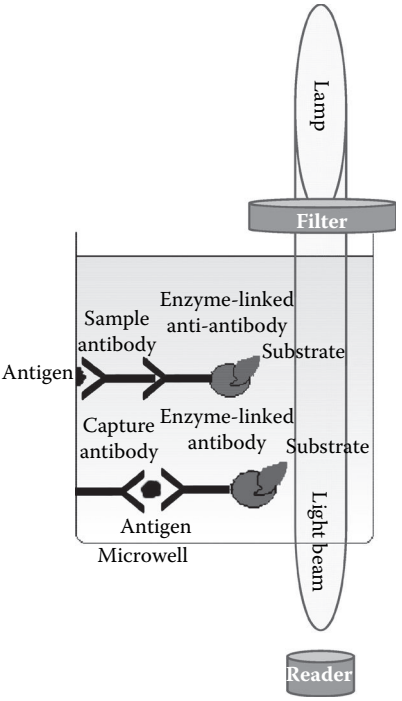


Figure 4.5 (See also color figure in the insert) Enzyme-linked immunosorbent assay (ELISA). The assay is performed in a multiwell microtiter plate. The antigen-antibody reaction is visualized by an enzymatic reaction, in which a colored product develops from a substrate. This is read photometrically and quantified as the reduction in light passing through the well, the optical density (OD), which is typically scaled from 0 to 4. If the aim is to diagnose the presence of the antigen in a sample, the capture ELISA is commonly used; that is, the surface of the microwell is coated with a capture antibody against the antigen. The capture of the antigen may then be directly visualized using another specific antibody conjugated with an enzyme, or indirectly by first using a specific antibody, and then an enzyme-conjugated anti-immunoglobulin (sandwich technique). If the aim is to demonstrate the presence of specific antibodies in a serum sample (i.e., serology), the surface of the microwell is coated with the antigen, and the enzyme-conjugated anti-immunoglobulin is used to demonstrate the antigen-antibody reaction. The method used for serology may also be used for demonstrating an antigen. In competitive ELISA, the sample containing the antigen is mixed with a serum sample with a known ELISA reaction, which then is tested by ELISA serology. A drop in the OD value is caused by antigen in the sample having used the antibodies in the serum.

Table 4.3 A Typical Protocol for Demonstrating the Presence of Bacteria in Samples by Enzyme-Linked Immunosorbent Assay (ELISA) (Direct Sandwich Method)

Materials

Specific antibody against the bacterium	Specific antibody against the bacterium conjugated with horseradish peroxidase (HRP)
Microtiter 8-well single strips (enhanced binding) + frame	2N sulfuric acid
Parafilm	Carbonate buffer (Table 4.5)
OPD (O-phenylenediamine) tablets	Washing buffer (Table 4.5)
10-ml glass tubes	Dilution buffer (Table 4.5)
Towel	Citrate buffer (Table 4.5)
Tinfoil	30% hydrogen peroxide (kept cold and covered by tinfoil)
2-ml cryotubes	
Positive control serum	Fetal calf serum

Equipment

Whirl mixer	ELISA reader
ELISA washer	Magnet stirrer
Multipipette with trays	

Coating the wells with the antibody

The antibody is diluted 1:1000 in carbonate buffer. The mixture is transferred to a multipipette tray. From the tray, 0.2 ml is added to each well. The plate is covered with parafilm and incubated at 4°C. After 24 h, the wells are emptied by banging the plate with the bottom upside down several times against a towel. The wells are filled with washing buffer with the plate washer and incubated 3 min at room temperature. The washing buffer is then removed, and the plate is banged against the towel. The washing procedure is repeated six times. All wells are then filled with the dilution buffer and incubated 2 h at room temperature (blocking); the six-step washing procedure is then repeated. After washing, the wells may be stored at –20°C until use if they are filled with the washing buffer.

Preparing the OPD chromogen solution

Four tablets are grappled with tweezers (*do not touch*) and placed in a small Erlenmeyer bottle. Then,12 ml citrate buffer and a magnet are added. The solution is stirred until the tablets are fully dissolved, then 5 µl 30% hydrogen peroxide are added. The glass is packed in tinfoil and used within 30 min.

Performing the assay

1. Add 0.2 ml test sample diluted 1:100 with the dilution buffer. A blind sample (dilution buffer only) and a known positive are added to each of two wells in each plate. The position of each sample is reported on a reporting sheet or computerized. The plate is incubated at room temperature for 2 h.

continued

Table 4.3 (continued) A Typical Protocol for Demonstrating the Presence of Bacteria in Samples by Enzyme-Linked Immunosorbent Assay (ELISA) (Direct Sandwich Method)

2. The samples are removed with the plate washer, and the plates are washed by the six-step washing procedure as described for the coating procedure.
3. In a proper dilution found at pretesting, 0.02 ml HRP-conjugated antibody is added to each well. The plate is incubated at room temperature for 2 h.
4. Repeat step 2.
5. To each well, add 0.2 ml OPD chromogen-solution (*safety cabinet*). The plates are incubated in the dark at room temperature for 10 min.
6. The reaction is stopped by adding 50 µl 2N sulfuric acid.
7. The plates are read with the ELISA reader. The 492-nm filter is used for reading, and the 630-nm filter is used as a reference.

antigen, one may coat single or double strips with 8 or 16 wells. Strips with different antigens can be collected in the same frame to make up a 96-well ELISA plate. The volume of the wells may vary from 50 to 250 µl. The higher the working volume, the lower the detection limit will be. Our preference is to work with 200 µl in 250-µl wells.

4.5.3 The antigen

In principle, bacteria may be used directly as antigen; that is, they are suspended in carbonate buffer (Table 4.5), and the well is coated with the buffer suspension. In most assays, it is advantageous to prepare the antigen for coating, minimally to prevent continuous growth. The simplest way is boiling the antigen for 75 min. Smaller particles may be achieved by sonication, and proteins may be extracted by the use of certain chemicals.

4.5.4 Antibodies, enzymes, and substrates

Enzyme-conjugated, species-specific anti-immunoglobulins are available from several commercial suppliers. They should usually be diluted in the range from 1:1000 to 1:4000. If this has not been determined by the producer, pretesting has to be done in the laboratory prior to use. Anti-immunoglobulins for some species may be difficult to find at commercial suppliers. This problem may be solved by testing an anti-immunoglobulin to a related species to identify if it binds acceptably to the immunoglobulin of the missing species; for example, antimouse may be applied when testing gerbils[8] (antigerbil is commercially available).

The most common conjugate enzyme is horseradish peroxidase, but alkaline phosphatase, β-galactosidase, and urease may also be applied. The substrate is, of course, specific to the enzyme. Furthermore, the substrate for ELISA has to be soluble. Table 4.6 lists enzymes, relevant substrates,

Table 4.4 A Typical Protocol for Performing Bacterial Serology by Enzyme-Linked Immunosorbent Assay (Indirect Sandwich Method)

Materials

Specific antigen	Specific anti-immunoglobulin conjugated with horseradish peroxidase (HRP)
Microtiter 8-well single strips (enhanced binding) + frame	2N sulfuric acid
Parafilm	Carbonate buffer (Table 4.5)
OPD tablets	Washing buffer (Table 4.5)
10-ml glass tubes	Dilution buffer (Table 4.5)
Towel	Citrate buffer (Table 4.5)
Tinfoil	30% hydrogen peroxide (kept cold and covered by tinfoil)
2-ml cryotubes	Fetal calf serum
Positive control serum	

Equipment

Whirl mixer	ELISA reader
ELISA washer	Magnetic stirrer
Multipipette with trays	

Coating the wells with the antigen

Resuspend 160 µg of the antigen in 0.5 ml carbonate buffer in a cryotube and whirl mix. The mixture is transferred to a multipipette tray to which 19.5 ml carbonate buffer are added. The suspension is mixed in the tray. From the tray, 0.2 ml is added to rows A, C, E, and G in a 96-well microtiter plate. To the other wells, only the carbonate buffer is added. The plate is covered with parafilm and incubated at 4°C. After 24 h, the wells are emptied by banging the plate with the bottom upside down several times against a towel. The wells are filled with washing buffer with the plate washer and incubated 3 min at room temperature. The washing buffer is then removed, and the plate is banged against the towel. The washing procedure is repeated six times. All wells are then filled with the dilution buffer and incubated 2 h at room temperature (blocking); the six-step washing procedure is then repeated. After washing, the wells may be stored at –20°C until use if they are filled with the washing buffer.

Preparing the OPD chromogen solution

Four tablets are grappled with tweezers (*do not touch*) and placed in a small Erlenmeyer bottle. Add 12 ml citrate buffer and a magnet. The solution is stirred until fully dissolved, then 5 µl 30% hydrogen peroxide are added. The glass is packed in tinfoil and used within 30 min.

Performing the assay

1. Add 0.2 ml test serum diluted 1:100 with the dilution buffer. Each test serum is added to an antigen-coated well and a control well (Figure 4.6). Positive and negative control sera are added in the same way in every plate. The position of each serum is reported on a reporting sheet or computerized. The plate is incubated at room temperature for 2 h.

continued

Table 4.4 (continued) A Typical Protocol for Performing Bacterial Serology by Enzyme-Linked Immunosorbent Assay (Indirect Sandwich Method)

2. The sera are removed with the plate washer, and the plates are washed by the six-step washing procedure as described for the coating procedure.
3. In a proper dilution found at pretesting, 0.02 ml species-specific, HRP-conjugated anti-immunoglobulin is added to each well. The plate is incubated at room temperature for 2 h.
4. Repeat step 2.
5. Then, 0.2 ml OPD chromogen solution is added to each well (*safety cabinet*). The plates are incubated in the dark at room temperature for 10 min.
6. The reaction is stopped by adding 50 µl 2N sulfuric acid.
7. The plates are read in the ELISA reader. The 492-nm filter is used for reading, and the 630-nm filter is used as a reference.

Table 4.5 Examples of Recipes for Buffers Used for ELISA

Carbonate buffer		**Washing buffer**	
Na_2CO_3, $10H_2O$	4.29 g	Tween 20	1 ml
$NaHCO_3$,	2.93 g	NaCl	15 g
Distilled water until	1000 ml	PBS until	1000 ml
Dissolve the carbonates in 800 ml water. Adjust pH to 9.6 with 1M HCl or NaOH. Dilute until 1000 ml. Store at 4°C.		Dissolve the Tween and salt in 800 ml water. Adjust pH to 7.8 with 1M HCl or NaOH. Dilute until 1000 ml. Store at 4°C.	
Dilution and blocking buffer		**Citrate buffer**	
Fetal calf serum	10 ml	Citric acid, H_2O	7.30 g
Tween 20	0.2 ml	Na_2HPO_4, $12H_2O$	23.88 g
PBS until	200 ml	Distilled water until	1000 ml
Dissolve the Tween and the serum in 800 ml water. Adjust pH to 7.2 with 1M HCl or NaOH. Dilute until 1000 ml. Should be used immediately or stored at –20°C.		Dissolve the acid and phosphates in 800 ml water. Adjust pH to 5.0 with 1M HCl or NaOH. Dilute until 1000 ml. Store at 4°C.	

and the chemical reaction they catalyze. Substrates are supplied by several commercial suppliers.

4.5.5 Coating the wells

Although absorption of protein to the plastic surface of the well is based on hydrophobic binding and thereby relatively independent of pH, a buffer with pH at or slightly above the pH_{ISO} is generally used, the most common one being a pH 9.6 carbonate buffer (Table 4.5). The buffer should

Table 4.6 Enzymes Used for Conjugation with Antibodies in ELISA and the Related Substrates, the Reactions Catalyzed, and the Color/Wavelength Produced during the Reaction

Enzyme		Substrate	Color/ wavelength	Chemical reaction
Horseradish peroxidase	TMB	Tetramethyl-benzidine hydrochloride	Yellow/ 450 nm	2 substrate-H + H_2O_2 → 2 substrate + 2 H_2O
	OPD	O-Phenylenediamine	Green/ 492 nm	
	ABTS	2,2′-Azino-di(3-ethyl-benzthizoline) sulfonate-6-diammonium salt	Green/ 410 nm	
Alkaline phosphatase	PNPP	P-Nitrophenylphosphate	Yellow/ 405 nm	PNPP → phosphate + P-nitrophenol
β-Galactosidase	ONPG	O-Nitro-phenylgalactoside	Yellow/ 420 nm	ONPG → galactose + O-nitrophenol

not contain any detergents because they will block the binding sites and thereby reduce the amount of antigen bound. The coating time should not be too short. Either 4 h at 37°C or 24 h at 4°C are generally usable. Care should be taken to ensure the same temperature in each well by placing the plates in a closed box (e.g., vaporization is avoided by covering the plates with parafilm). The coating time may be reduced if the plates are shaken on a plate shaker during coating. To eliminate the impact of unspecific binding on the interpretation of the result, it is an option to coat rows with both antigen and pure buffer within the same plate (Figure 4.6).

4.5.6 Blocking the wells

Adding Tween 20 to the dilution buffer is necessary to avoid unspecific binding on nonantigen-covered plastic. Tween 20 alone may, however, be insufficient; therefore, some kind of nonimmunoglobulin-binding protein is generally used as a blocking agent. The blocking agents are added to the dilution buffer in a concentration typically of 5%. The most common blocking agent is bovine serum albumin (BSA), but various types of serum are also used. To avoid specific antibodies, fetal calf serum is preferable when using serum for blocking. Alternatively, cow milk is a cheap but reliable option. A blocking step, in which the wells are filled with the dilution buffer with 5% blocking agent, should be introduced in the protocol after coating but prior to testing the samples.

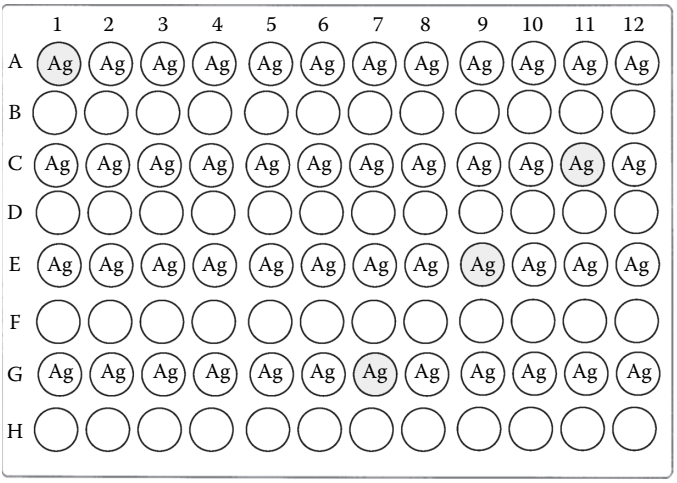


Figure 4.6 A system for setting up a 96-well microtiter plate for ELISA. Only half of the rows (A, C, E, and G) are coated with the antigen; the remaining rows (B, D, F, and H) during the coating step are filled with the pure coating buffer only. A1 and B1 are filled during the test with a positive control serum, and C1 and D1 are filled with a negative control serum. Test sera are filled into each coated and non-coated well. A high OD value in A1 is expected, while B1, C1, and D1, as well as all wells in rows B, D, F, and H, should have OD values far below the cutoff value.

4.5.7 Performing the assay

A typical protocol for capture ELISA is shown in Table 4.3 and for ELISA serology in Table 4.4. The method must be optimized individually from laboratory to laboratory and from test to test. Having optimized all of the factors mentioned, such as the type of plates, the antigen, and so on, the temperatures and times of incubation still have to be considered. Incubation temperature may range from 4°C to 40°C, but in practice, there is a choice between 4°C (refrigerator), 20°C (room temperature), and 37°C (incubator). The highest achievable enzymatic activity is not always optimal, as this also leads to more reactions that are nonspecific. Likewise, the incubation time has to be considered. The longer the time, the more antigen-antibody reactions there are, but also the more unspecific reactions there are. At a certain level, no more antigen-antibody reactions will occur as a result of increased temperature or incubation time, and in general, an increase in temperature means a shorter incubation time. For example, the same result might be achieved at 4°C for 24 h, at 20°C for 2 h, and at 37°C for 30 min. Also, shaking the plates on a plate shaker enhances the antigen-antibody reactions and might thereby reduce the incubation time.

4.5.8 Control sera

Control sera may derive either from spontaneously infected animals or from immunized animals. Sera from germ-free animals are directly usable as negative controls. Positive control sera should be pretested in different dilutions (e.g., 1:25, 1:50, 1:100, and 1:200), and a dilution giving an optical density (OD) value within an acceptable and usable range (e.g., between 1 and 2.5) should be chosen as the standard for that serum. Commercially available control sera may be diluted according to instructions given by the supplier, but pretesting at lower dilutions in the laboratory is recommended. For all dilutions, the dilution buffer (Table 4.5) is used. Control sera may be stored at –20°C, but they must be stored in small volumes because they should be thawed only once.

4.5.9 Interpretation of the OD value

As the main aim of routine health monitoring of laboratory animals is to diagnose the infection on the colony level and not on an individual level, it is highly recommended to use high cutoff values for bacterial serology and counteract the drop in sensitivity by sampling more animals. If noncoated wells are included for every sample, the value used for interpretation of a certain serum should be the difference between the OD value of the coated and noncoated wells inoculated with that serum. To eliminate day-to-day variation, it is recommended to include a standard serum in the assay. Prior to interpretation, all OD values are divided by the OD value of the standard serum, and the ratio is used for interpretation. This presupposes that a certain amount of standard serum is available and that the OD value of this supply does not decline over time, which is not always the case. Therefore, the use of ratios is mostly applicable in projects run over a short period. One way to set a cutoff value is to choose a value much lower than the lowest value found in a number of positive samples tested (e.g., 0.2) and subsequently optimize the assay to ensure that none of a high number of negative samples show values higher than the cutoff value. Table 4.7 shows another system based on statistical calculation on a number of known negative samples.[9]

4.6 Immunoblotting

Antibodies may be detected by so-called immunoblotting.[10] In *Western blotting*, proteins of the bacterium are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and are thereafter transferred to an immobilizing membrane, in which they are irreversibly bound. Transfer may be done in different ways (e.g., by the buffer tank blotting technique) (Figure 4.7). If the proteins are placed directly on the

Table 4.7 A System for Setting Cutoff Values When Using ELISA Serology in Laboratory Animal Health Monitoring

Negative	$\text{Mean}_{\text{negatives}} + 4 * \text{SD}_{\text{negatives}} > \text{Sample OD}$	$p \geq 0.0313$
+	$\text{Mean}_{\text{negatives}} + 4 * \text{SD}_{\text{negatives}} \leq \text{Sample OD} > \text{mean}_{\text{negatives}} + 8 * \text{SD}_{\text{negatives}}$	$0.0313 > p \geq 0.0078$
++	$\text{Mean}_{\text{negatives}} + 8 * \text{SD}_{\text{negatives}} \leq \text{Sample OD} > \text{mean}_{\text{negatives}} + 12 * \text{SD}_{\text{negatives}}$	$0.0078 > p \geq 0.0035$
+++	$\text{Mean}_{\text{negatives}} + 12 * \text{SD}_{\text{negatives}} \leq \text{Sample OD}$	$0.0035 > p$

Note: The mean and the standard deviation of the OD values found in a number of samples from noninfected colonies are used for estimation of a cutoff value according to Chebyshev's theorem. The p value describes the highest possible probability that a sample OD value within a given range should belong to the same normal distribution as the negative samples pretested.

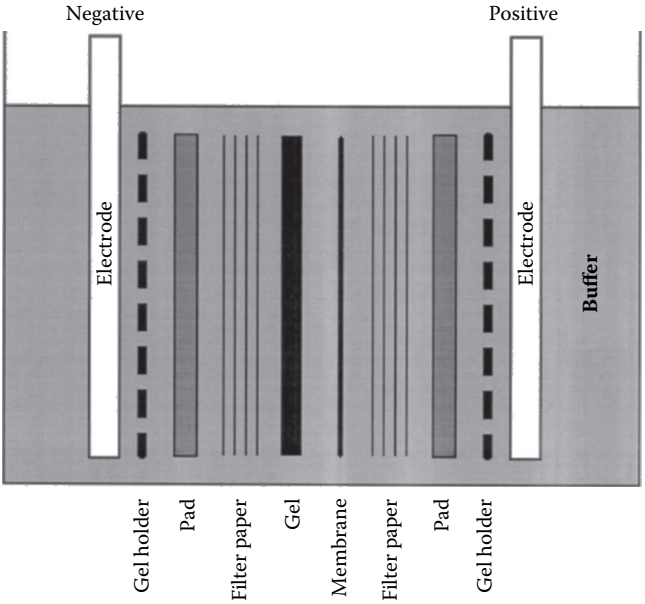


Figure 4.7 The buffer tank blotting technique. Proteins are transferred from an electrophoretic gel to an immobilizing membrane, in which they are irreversibly bound.

membrane, the technique is called *dot blotting* instead of Western blotting. The membrane is made of nitrocellulose or, alternatively, polyvinylidene chloride or nylon if the chemiluminescent detection technique is to be applied. After blocking free-protein sites, the test serum is added, and antigen-antibody reactions with the proteins are visualized by using fluorescein-, enzyme-, or radionucleotide-labeled anti-immunoglobulin. In

laboratory animal bacterial serology, the method has not been as widely used as IFA and ELISA.

4.7 Multiplex

In multiplex assays, several antigens are tested simultaneously; that is, multiplex assays can be applied for the entire health-monitoring profile in one run when screening rodent colonies, in contrast to the other methods described, in which one assay per antigen is performed. In the *Luminex® bead-based assay*, beads are stained with dyes with a specific spectrum for each bead, and each bead is coated with a specific antigen. All beads are mixed together in a well with the diluted test serum. After washing, biotinylated species-specific anti-antibodies are added and form an antibody-antigen sandwich in the same way as shown for the labeled avidin-biotin method in Figure 4.4. After yet another washing step, an enzymatic reaction may be made, by adding an avidin-conjugated enzyme, then still another washing step and then the enzyme's substrate. After a final washing step, the beads are passed through a dual-laser flow-based reader as shown in Figure 4.8. The Luminex® technology enables up to 500 antigens

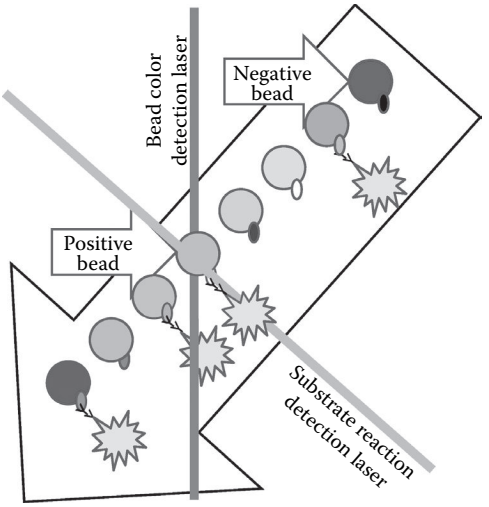


Figure 4.8 (See also color figure in the insert) Principles of reading the Luminex® bead-based multiplex assays. Sandwich reactions are made between the antigen and the test serum in a well. Each antigen is linked to a bead with a specific color; therefore, several antigen-antibody reactions can be performed in the same well. If the sandwich reaction (see Figure 4.6) takes place, the bead has been bound to an enzyme that will catalyze a color-forming reaction with a substrate. When flown through the reader, one laser beam will identify the bead, and another laser beam will check for the reaction.

to be detected in each well of a 96- or 384-well plate, using very small sample volume. The method is used often in research for quantification (e.g., of cytokines and metabolic markers in laboratory rodents), and it has been applied for some time in human diagnostic bacteriology.[11] It has now also taken its place as a routine method in larger commercial and vendor laboratories for rodent health monitoring.

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chapter five

Molecular biology-based methods for microbiota characterization

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5.1 Why molecular biology-based methods?

The rodent gut microbiota (GM) consists of several hundred different species, with different growth requirements, meaning that an enormously wide range of different substrates will be necessary for their cultivation.[1] Furthermore, the majority (60–80%) of the microbial gut inhabitants cannot be cultivated using existing methods/protocols.[2] This is probably because some species are extremely oxygen sensitive, others have specific growth requirements, and yet others most likely proliferate in a symbiotic relationship, in which they rely on other species to supply them with essential nutrients. Furthermore, as some species are several orders of magnitude more abundant than others in the rodent GM, it would be necessary to pick, purify, and identify an enormous amount of isolates to obtain an in-depth overview of the GM composition should substrates supporting the growth of the majority of GM members one day be developed.[2]

The enormous development within tools and techniques based on molecular biology for culture-independent characterization of complex microbial societies has consequently been essential for the much deeper understanding of the complex interplay between host, environment and GM during recent years.[3–5] Furthermore, it has led to an increased understanding of how the GM influences the reliability of animal models for the study of human diseases.[6–8]

Here, a brief introduction is given to the different molecular biology-based, culture-independent methods that are most widely used for GM characterization.

5.2 RNA and DNA storage and extraction

Molecular biology-based, culture-independent methods are based on the informational molecules RNA and DNA. In general, DNA is targeted for microbiota characterization, although also the transcriptome is receiving increasing interest,[4] while RNA is the target when analyzing gene expression in, for example, tissue or epithelial samples. Storage of samples in conditions under which these molecules are kept intact is thus essential. In general, samples should be quickly frozen and stored at as cold a temperature as possible. For RNA, addition of, for example, RNAlater® (many different suppliers offer suitable solutions) followed by snap freezing in liquid nitrogen are preferred. Long-term (months) storage is then possible in liquid nitrogen or at −80°C. DNA is more stable than RNA, and rapid freezing is consequently not as important. Nevertheless, for storage longer than a couple of days, temperatures above −40°C are to be avoided; in general, −80°C or colder is preferred. Reliable and reproducible extraction of RNA or DNA is the next important step. Commercial suppliers offer a wide range of solutions, such as the Stool Kit marketed by Qiagen (Valencia, CA, USA) (publications indicate use in hundreds of experiments targeting human or rodent fecal samples) and the PowerSoil kit (MoBio Laboratories, Carlsbad, CA, USA), used by (among other projects) the Human Microbiome Project (HMP) in the United States.[9] Other solutions include, for example, classical phenol/chloroform extraction-based methods, such as the protocol used in the European Union-financed MetaHIT project. In all cases, even and preferably complete lysis of microbial cells is essential. Introducing a bead-beating step for mechanical cell disruption before enzymatic/chemical lysis of cells is preferred by many groups.[10]

5.3 Denaturing and temperature gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are techniques based on sequence-specific separation of ribosomal RNA (rRNA) gene amplicons derived by polymerase chain reaction (PCR) using polyacrylamide gel electrophoresis (Figure 5.1A–5.1C). The PCR fragments have similar size but differ in sequence and, consequently, have different melting properties.[11] In DGGE, the gels contain a linearly increasing concentration of denaturant (urea and formamide). As the PCR fragments migrate through the gel, they

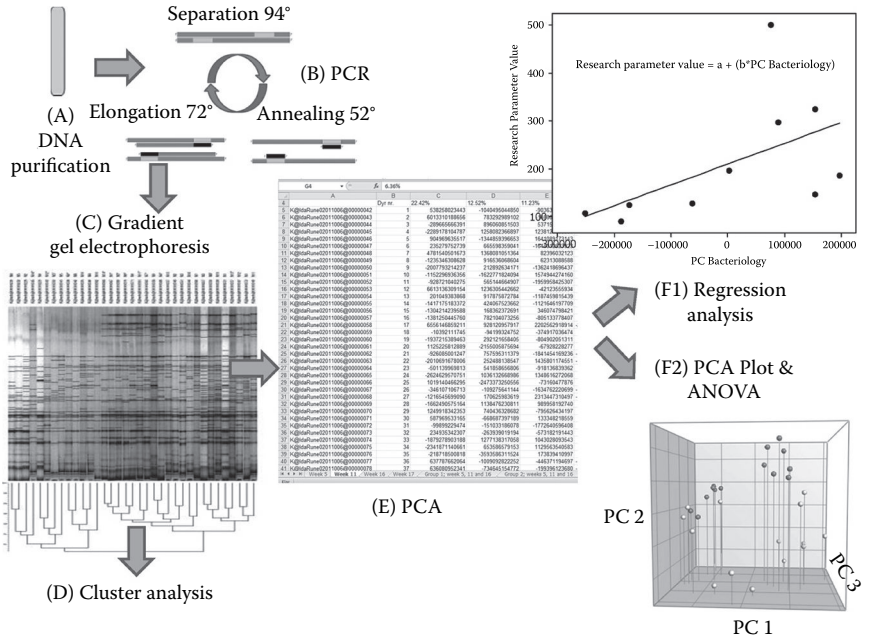


Figure 5.1 The use of gradient gel electrophoresis (GGE) for microbiota characterization. The bacterial DNA is purified (A) and amplified with polymerase chain reaction (PCR) (B). The PCR products are separated on a polyacrylamide gel (C) with either a chemical denaturation gradient (DGGE) or a temperature gradient (TGGE), resulting in each individual sample consisting of several bands representing specific groups of bacteria. The individuals can be sorted according to similarity, and the similarity coefficient between individuals can be calculated and shown as a cluster analysis for the entire group of sample (D). The differences on those parts of the plot explaining the largest amount of variance (principal component [PC] 1), second most variance explained (PC 2), and third most variance explained (PC 3), and in principle even further PCs can be quantified in a principle component analysis (PCA) (E), which can be used to correlate one of the PCs to a quantitative response in the animal model (F1) or to plot the values in a three-dimensional plot showing, for example, the test mice as dark bullets and control mice as light bullets and eventually comparing these by analysis of variance (ANOVA) (F2).

encounter increasing concentrations of denaturants, causing them to denature, which lowers their mobility and allows them to be separated as discrete bands based on their melting properties.[11] In TGGE, the same principle is applied, but the PCR amplicons migrate through a temperature gradient instead of a chemical gradient.[11] In principle, each band represents one taxonomic entity, but as most bacteria contain more than one copy of the *rrn*-operon encoding the rRNA genes and in many cases with

slight sequence differences between the *rrn*-operon copies, one strain or species might result in more than one band, leading to overestimation of microbial diversity.[12] Similarly, one band might contain PCR amplicons representing two or more different species with identical melting properties,[13] which will lead to underestimation of microbial diversity.

DGGE and TGGE have been widely used to study mammalian GM composition since the early 2000s, as important tools, especially before development of the high-throughput sequencing (HTS) techniques, offering a relatively detailed picture of complex microbial societies at a relatively low price.

The 16S rRNA gene is normally targeted when investigating bacterial communities, whereas either the 18S or the 26S rRNA (D1-region) genes are usually preferred if fungal and yeast communities are targeted. For bacteria, a range of "universal" prokaryotic primer sets targeting different variable regions of the 16S rRNA gene are available, including the V1, V1-V3, V3 and V6-V8 regions. Primers targeting the V3 region in general give the highest resolution (determined as number of bands).[14] It is important to remember that universal primers are not truly universal,[15] and choice of primers thus influences which species will be detected or not. Furthermore, using universal primers, only the most abundant species (estimated to about less than 1% of the total population) will be detected. If less-abundant rodent microbiota members such as archaea,[16] lactic acid bacteria,[17–19] or *Bifidobacteria*[12] (the last two, for example, in studies investigating the effect of probiotics) are of particular interest, primers specifically targeting these groups should be used.[20]

If needed, the separated bands of interest can be excised from the gels, reamplified and sequenced to identify the microorganisms present to at least genus, often even species level.[11,14,21] Unfortunately, this process is in most cases tedious as the excised fragments often contain not only DNA from the target sequence but also DNA from a background smear of single-stranded DNA that also will be amplified during PCR, giving mixed sequences. Before sequencing, it is thus essential to check the electrophoretic mobility and purity of the reamplified fragments by a new DGGE/TGGE run.[14,22] As the process of identifying bands of interest by sequencing is tedious, and as the cost for HTS-based community characterization is rapidly dropping, sequencing of bands is rarely done currently. However, should it be desired to identify DGGE or TGGE bands by sequencing, Zhang et al.[22] suggested several ways to purify the excised fragments before reamplification, such as treatment with mung bean nuclease.

DGGE and TGGE gels may vary from run to run, which makes comparing profiles from different gels challenging. However, by including a suitable standard (e.g., a mixture of amplicons representing known species with melting properties that span the whole gel) and using gel analysis

software (such as BioNumerics [Applied Maths, Sint–Martens–Latem, Belgium]), this problem can easily be overcome by normalizing the gels against the standard.[14] Furthermore, Nielsen et al.[23] demonstrated that tools originally developed for aligning and analyzing high-performance liquid chromatographic (HPLC) chromatograms such as baseline correction and correlation-optimized warping (COW) can be useful data pretreatment tools for DGGE analysis. After normalization, cluster analysis and principal component (PC) analysis are useful tools for visualizing data structures (Figure 5.1D–E). An advantage of PC analysis is that the PC coordinates can be exported and used for investigating correlations to, for example, physiological parameters (Figure 5.1, F1), or they can be compared statistically between groups (Figure 5.1, F2).[10]

5.4 *Terminal restriction fragment length polymorphism analysis*

Terminal restriction fragment length polymorphism (T-RFLP) is based on PCR-based amplification of a target gene (often one of the rRNA genes) using primers for which one of the primers is fluorescently labeled (Figure 5.2). The resulting amplicons will then be fluorescently labeled in one end. The PCR amplicons are digested with one or several restriction enzymes followed by separation of the digested fragments on an automated sequencer using capillary gel electrophoresis. Only the labeled terminal ends of the fragments (referred to as TRFs, terminal restriction fragments) will be detected.[24] The length heterogeneity of the detected TRFs indicates the complexity of the sample, which is visualized on an electropherogram (Figure 5.2) with the *x* axis indicating the size of the fragment and the *y* axis the intensity ("abundance"). Tentative identification of the detected TRFs can be achieved by comparing the size of the TRF to either a known clone library or databases such as the Ribosomal Database Project (RDP), given that the 16S rRNA gene is the target gene.[25,26] The T-RFLP method has the same advantages and limitations as DGGE and TGGE, also in terms of PCR bias and the like. However, T-RFLP has higher throughput compared to DGGE/TGGE; the major limitation of T-RFLP is the lack of possibility to sequence peaks of interest.[26] As with DGGE, T-RFLP is progressively being replaced with HTS-based techniques but remains in use in many laboratories.

5.5 *Clone library analysis*

In targeted clone library analysis, a target gene, usually one of the ribosomal genes, is amplified, and the amplicons are cloned and subsequently sequenced using traditional Sanger sequencing. This approach

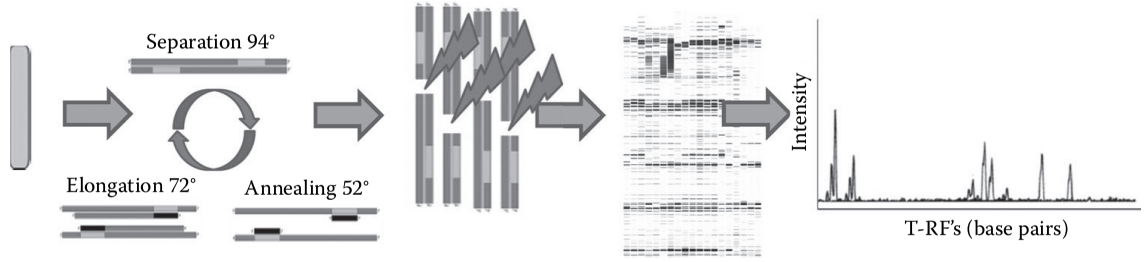


Figure 5.2 Creation of an electropherogram by analysis based on terminal restriction fragment length polymorphism (T-RFLP). The gel reads can be used for cluster and principal component analysis and subsequent analysis of variance (ANOVA) and regression as described in Figure 5.1.

has previously been widely used to study, for example, human, mouse, and pig GM composition.[27–29] Clone library analyses yield high-quality, long-read sequences, but at a rather high cost and have been almost totally replaced by HTS-based technologies.

5.6 High-throughput sequencing

In 2005, the first high-throughput sequencing (HTS) platform (i.e., the 454 GS20 pyrosequencing platform developed by the Roche company 454 Life Sciences, Branford, CT, USA) became commercially available. Since then, HTS has seen continuous development and improvement of technologies, offering more and longer reads at a lower and lower price.[30–32] A range of different HTS platforms has been developed, such as 454/GS FLX (454 Life Sciences); HiSeq (Illumina, San Diego, CA, USA); MiSeq (also Illumina); SOLiD (Life Technologies, Grand Island, NY, USA); Ion Torrent (also Life Technologies); Helicos (Helicos Biosciences, Cambridge, MA, USA [out of business]); and PacBio (Pacific Biosciences, Menlo Park, CA, USA). The 454/GS FLX Titanium platform yields around 1 million reads with a length of 450–700 nucleotides, and, for example, the SOLiD platform offers 150 million reads (read length of 35–50 nucleotides)[30,32,33] and the HiSeq 2500 platform offers more than 1 billion reads with a read length of about 160 bp (2 × 100 bp).[31] At present, the HiSeq platform is the platform of choice for most laboratories doing full-metagenome analysis of the gut microbiome, for example, as exemplified by a number of high-impact publications.[34–36] The Ion Torrent Proton platform performs well, giving somewhat fewer reads but much shorter sequencing time compared to the HiSeq platform.[31] Full-metagenome sequencing is the gold standard in culture-independent microbiome analysis, but it has two major drawbacks. The first is price, with a price per sample of around US$1400; the second is data analysis. Even in relatively modest experimental designs, massive amounts of sequencing data will be produced, and data analysis almost inevitably becomes a bottleneck unless significant resources in terms of both computing and manpower are used.

Amplicon sequencing offers a cheaper (and data analysis-wise more feasible) alternative to full-metagenome sequencing. The first step in amplicon sequencing is PCR-based amplification of a target gene. Often, a fragment of the 16S rRNA gene (with the V3-V4 region used in many studies) is targeted. Long read lengths are necessary, and for several years the 454/GS FLX platform was the platform of choice for amplicon sequencing, but the MiSeq (especially after the recent release of 250- and 300-bp paired end kits) and Ion Torrent PGM technologies now offer the best solutions for this, with the PGM being faster, but the MiSeq platform offering more reads for the money.[31] To bring down the cost per sample, the 3–15 million

reads/run offered by these technologies can be divided between several samples by tagging different samples with unique tags during PCR at less cost, but often adequate sequencing depth. Subsequently, the tagged fragments are pooled, sequenced, sorted according to tags, and analyzed.[1,37]

Several platforms have been developed for analyzing rRNA gene-targeted amplicon sequences. Figure 5.3 illustrates a typical workflow. At present, the QIIME (http://qiime.org/; Quantitative Insights Into Microbial Ecology) platform is preferred by many groups,[38] but the analytical techniques are constantly being improved, and it is advised always to consult the most recent literature and Internet-based HTS user communities for which tools to use before starting analyzing data. The recently developed

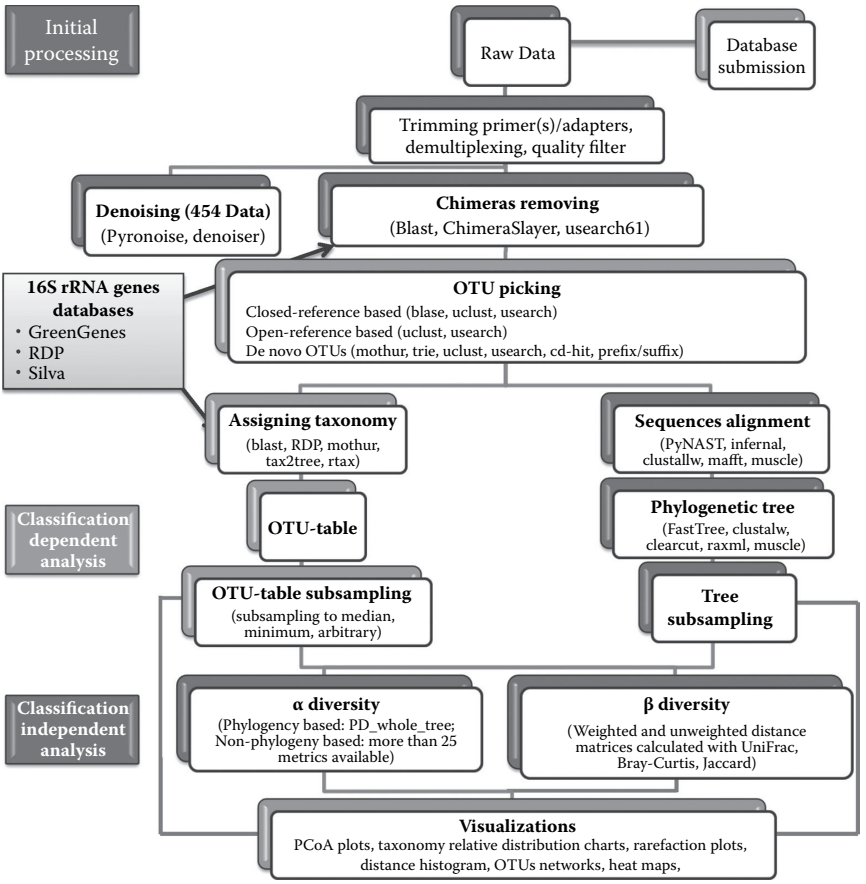


Figure 5.3 Typical workflow and output when analyzing high-throughput tag-encoded 16S rRNA gene amplicon sequences. (Courtesy of Lukasz Krych, University of Copenhagen.)

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) tool offers the possibility to predict the functional composition of a microbiome using marker data, such as 16S rRNA gene amplicon data.[39]

Pacific Biosciences has recently started marketing their PacBio platform. This platform offers read lengths up to as much as 10,000–20,000 bp, but, unfortunately, it is at the cost of fewer reads (i.e., 20,000–60,000 reads per run). Furthermore, the PacBio platform has higher error rates than other HTS technologies, but because of the exceptionally long reads, the technique nevertheless holds great promise for the future because of the long read length and the possibility to sequence single molecules.[31,32]

5.7 Quantitative real-time PCR

Culture-independent methods like DGGE, TGGE, and T-RFLP yield qualitative (yes/no or present/not present) results. HTS offers information on the relative abundance of different operational taxonomic units but does not offer absolute quantification. For culture-independent quantification of, for example, specific species in the gastrointestinal tract (or any other environment), quantitative real-time PCR (qPCR) is the method of choice. qPCR is carried out using a thermocycler equipped with a fluorescence detector. During PCR, the amount of DNA generated is continuously monitored either by incorporating sequence-specific fluorescence-emitting probes into the PCR products or including a dye such as SYBR Gold that emits fluorescence when bound to double-stranded DNA. With increasing numbers of PCR cycles, more and more PCR product is produced; therefore, the signal also increases. Using a calibration curve based on a serially diluted standard with known concentration, this signal can be linked to the exact amount of template in the sample.[40,41] Depending on the primers used, qPCR can detect and quantify taxonomic and functional genes all the way from kingdom to genus, species, and in some cases even strain level. This obviously makes qPCR an extremely versatile method that can be used for gene expression studies as well as enumerating groups of organisms.[37,40–42] Dead cells can be distinguished from viable cells either by extracting RNA (followed by reverse transcription before qPCR) or by so-called EMA-qPCR where DNA from dead cells is irreversibly bound to ethidium bromide monoazide (EMA), which will prevent amplification of DNA from dead cells.[43] The different steps from RNA/DNA extraction over primer design and use of calibration curves contain many pitfalls that all will influence the outcome of the analysis. See, for example, the review by Smith and Osborn[41] for a more thorough discussion of this. Specific qPCR-based assays for quantification of specific bacteria such as *Akkermansia muciniphila* offer valuable tools to confirm and expand the

findings of HTS-based studies, for example.[42] Several qPCRs for a range of different bacteria species or groups of bacteria may be combined to make a detailed microbiota characterization.[44]

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chapter six

Mechanisms behind bacterial impact on animal models

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6.1 Mechanisms of pathogenicity and research interference from specific bacteria

6.1.1 Pathogenicity versus research interference

In veterinary medicine, the traditional reason for showing interest in specific infectious agents has been their ability to cause disease, that is, their *pathogenicity*. This is, of course, also vital in laboratory animal medicine, as only healthy animals should be used for experiments. However, the impact of infections goes far beyond this: Infections may interfere with research, even in the absence of clinical symptoms or pathological changes. Specific bacteria may have a direct impact on the immunology, the physiology, the reproduction, other parts of the microbiology, and the oncology.[1] Even if the study includes a test group and a control group, infections are still important because specific bacteria

- may inhibit the induction of a certain animal model.
- may make it difficult to interpret the final results.
- may be dose related in their effect if the test factor acts as an inducer.
- may increase the variation inside the group, thereby leading to the use of more animals.

The interference may also be more indirect, such as by the contamination of biological products used for experiments. Some microorganisms only influence the animal temporarily; others—and that certainly goes for a range of bacteria—act over a long period, perhaps even for the entire life of the animal. This division is mainly connected with the ability of the agent to persist in the organism. This is, however, not always so, as can be illustrated by the very simple example given by those infections that induce resistance against reinfection. The agent leaves the organism after causing a permanent change in the immune system. Such examples also illustrate that a microorganism interferes with a certain part of the animal permanently, in this case, the immune system; other parts of the animal (e.g., its anatomy) are only affected temporarily. If an animal colony is infected with microorganisms that only temporarily influence the individuals, this influence may only be observed in animals of a certain age, and many studies will not be influenced. Other studies may incidentally be designed in such a way that they use exactly those animals that received interference.

6.1.2 *Epidemiological terms*

In a broad sense, the word *infection* is used within laboratory animal medicine to cover the presence of a microorganism—the *agent*—in both an animal host and in a colony including several hosts. As the aim of performing examinations for specific bacteria within a health-monitoring program for laboratory animals is more in the direction of preventing microbial impact rather than diagnosing it after it has already occurred, the status of the colony in most cases is more important than the status of the individual. Terms used to describe infections on different levels are given in Table 6.1.

6.1.3 *Host–bacterial interaction influencing pathogenicity and research interference*

Animal susceptibility to the development of disease is controlled by genetics, sex, sexual cycle, age, and other characteristics of the host. Obviously, immune-deficient animals are more susceptible to the development of infectious disease, such as the observation of periorbital and preputial

Table 6.1 Terms Used in Laboratory Animal Epizootiology

Agent	The causative organism in a specific infection
Association	The presence of a symbiont or commensal in an animal host
Commensal	Microorganism that has no impact on the host organism
Copathogen	A microorganism-enhancing disease primarily produced by another agent
Determinants	All factors, the agent included, involved in the development of disease
Disease	Clinical manifestation of infection
Monofactorial disease	Disease in which the agent is the only factor influencing whether disease is observed
Multifactorial disease	Disease that is the result of several factors, including the agent
Health status	The actual status of an individual animal concerning its clinical, pathological, and physiological appearance
Inapparent carrier	The animal harboring a subclinical infection
Incidence	The fraction of new cases of infection over a defined period
Infection	The presence of a microorganism in an animal host or in an animal colony
Subclinical infection	Infection without clinical manifestations
Dormant subclinical infection	Subclinical infection in which the agent can be recovered
Latent subclinical infection	Subclinical infection in which the presence of the agent can only be proved by indirect methods
Inducer	Determinant enhancing disease in the animal
Infectivity	The ability of the microorganism to infect individuals
Microbiota	The entire ecosystem of both eukaryotic and prokaryotic organisms in a specified host or organ
Microflora	The entire ecosystem of bacteria in a specified host or organ
Opportunist	Facultative pathogen
Pathogen	A microorganism capable of inducing disease
Obligate pathogen	Microorganism always causing disease
Facultative pathogen	Microorganism only causing disease in connection with inducers
Pathogenicity	The ability of a certain microorganism to cause disease
Pathogenic	Capable of causing disease

continued

Table 6.1 (continued) Terms Used in Laboratory Animal Epizootiology

Prevalence	The fraction of animals infected with a certain microorganism at a certain point of time
Protector	Determinant protecting against disease in the animal
Saprophytic	Incapable of causing disease
Symbiont	Microorganism that has a positive value for the host organism
Virulence	A quantitative measure of the pathogenicity of the agent

abscesses containing *Pasteurella pneumotropica* in nude[2] and HIV-1 transgenic mice.[3] Inbred strains of rats are known to show variation in susceptibility to the development of diseases, such as Tyzzer's disease.[4,5]

Bacterial genes code for differences in virulence. The application of good hygienic principles will reduce the number of animals exposed to a lethal dose of bacteria, as there often is a gap between the infective dose and the lethal dose. For example, in guinea pigs the infective dose of *Bordetella bronchiseptica* has been found to be 4 colony-forming units, while the lethal dose has been found to be 1314 colony-forming units.[6] Some bacteria enhance the impact of other bacteria; for example, respiratory disease caused by *M. pulmonis* will be enhanced by the presence of *P. pneumotropica*,[7] which in this case makes *P. pneumotropica* a copathogen rather than an autonomous pathogen.

6.1.3.1 Adhesins and receptors

To become part of the host microbiota or to be able to act as a pathogen, bacteria need to be able to colonize the host. To do so, part of the bacteria, known as adhesins or bacterial ligands (Figure 6.1), must attach to parts of the host (i.e., host receptors). These structures most likely have other functions in the bacteria and the host, respectively; for example, in the bacteria, the same structures may act as epitopes, building blocks, and functionalities, such as motility. Gram-negative bacterial fimbria, or rather smaller proteins at their tips, function as adhesins, and in Gram-positive bacteria, adhesins are proteins or polysaccharide surface layers.[8] This adhesion-ligand system is essential for defining the tissue tropism and host specificity of certain bacteria (e.g., as it is known for *Clostridium piliforme*[9]), and the possession of adhesins is seen as a key—although not the only—element in bacterial virulence.[8]

Lectins are important: They bind to sugars linked to either proteins (glycoproteins) or lipids (glycolipids). Both are found in both mammals and in bacteria; therefore, they may act as both adhesins and receptors. An example of lectin-driven bacterial virulence can be given by the lectins of *Pseudomonas aeruginosa*, PA-IL, which binds to complexes containing

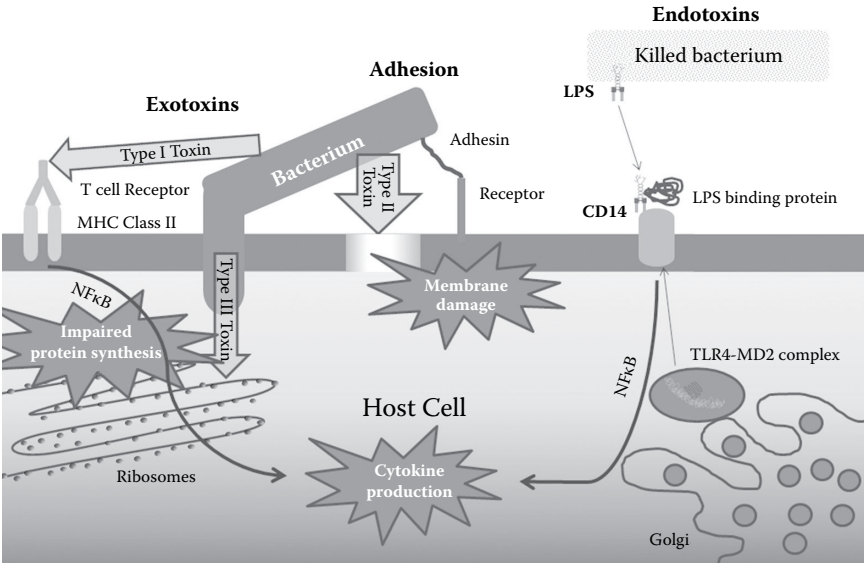


Figure 6.1 (See also color figure in the insert) Mechanisms behind bacterial pathogenicity. The bacterium will adhere to the cell receptor through an adhesin. This binding is likely to be lectin-sugar binding. Type I exotoxins (superantigens) will act on the T-cell exoreceptor, type II exotoxins will act on the cell surface, and type III exotoxins will be injected into the cell to act on protein synthesis. Other toxin mechanisms may also be observed. If a Gram-negative bacterium is killed, lipopolysaccharides (LPSs) will be released and presented for Toll-like receptor 4 (TLR4) via CD14. Cytokine production is mediated through the transcription factor nuclear factor kB (NFkB).

galactose,[10] and PA-IIL, which binds to complexes containing either fucose or mannose,[11] thereby facilitating bacterial adherence to surface epithelia, causing defects in the cellular barrier and allowing the penetrance of toxins.[12] One important binding site in animals is the α-gal epitope,[13] but humans and nonhuman primates lack the α-gal-transferase that produces this epitope. Therefore, the fucose-containing Lewis antigens are important for development of fatal *P. aeruginosa* pneumonias in humans[14]; these antigens are upregulated in patients with cystic fibrosis, thereby enhancing disease development.[15] Mice do express some fucose-binding receptors in their lungs,[16] but in general, in most mammals the α-gal-epitope dominates. This epitope is hardly expressed in the lungs of mice,[12] while mink and other stoats are the only animal species known to spontaneously develop *P. aeruginosa* pneumonias, efficiently bind both PA-IL and PA-IIL in their lungs, and therefore probably are superior to the mice as models of human cystic fibrosis pneumonia.[17]

Another receptor is glycoprotein 2 (GP2), which is found in the M (microfold) cells of Peyer patches and binds to FimH, a component of type I pili on the outer membrane of bacteria, such as *Escherichia coli* and *Salmonella typhimurium*.[18] This allows phagocytosis and, for nonpathogenic species, presentation of their antigens for the dendritic cells[19]; toxin production of pathogenic bacteria (e.g., *Salmonella*) interferes with this.[20] The host protects itself against these bacteria by secreting FimH-binding GP2 in the pancreatic juices.[21]

6.1.3.2 Toxins

Toxins are organochemical products either actively secreted by bacteria (exotoxins) or released from the bacteria when these are killed (e.g., because of reactions of the immune system [endotoxins]). Exotoxins are attack host cell structures to promote invasion and prevent attacks from the immune system.

Type I exotoxins (Figure 6.1) bind to a receptor on the cell surface to stimulate intracellular signaling pathways. In this group, we find the so-called superantigens (e.g., those produced by *Staphylococcus aureus*), which bind to antigen-presenting cells (APCs; see Section 6.2.1) and T cells, triggering the proliferation of the targeted T lymphocytes, releasing a high amount of various cytokines and other effectors, leading to toxic shock.[22] Another group of Type I exotoxins are heat-stable enterotoxins, which are produced by *E. coli*, but as colibacillosis in laboratory animals is mostly observed in rabbits, these toxins are less relevant because rabbit *E. coli* are not enterotoxin producing.[23]

Type II exotoxins (Figure 6.1) are membrane damaging at high concentrations; at lower concentrations, they may exhibit more subtle effects, such as modulation of host cell signal transduction without cell lysis. Some of these, such as those found in *S. aureus* and *Mycobacterium tuberculosis*, form pores in the target cell membrane[24]; others have an enzymatic effect on the membrane, such as the α toxin of *Clostridium perfringens*, which plays a role in the development of epizootic rabbit enteropathy.[25]

Type III exotoxins (Figure 6.1) exert their effect inside the cell (e.g., as is the case for many pathogenic Gram-negative bacteria). These are important for the pathogenicity of *P. aeruginosa*, which in mouse models of cystic fibrosis forms a proteinaceous channel that is inserted into the host cell membrane for injection of bacterial proteins that manipulate host cell signaling.[26] AB toxins use their B unit for binding to cell membranes, while the A unit enters through the membrane and exhibits an enzymatic function that affects internal cellular mechanisms, such as heat-labile *E. coli* toxin does.[27] Inside the host cell cytoplasm, some of the exotoxins, such as *Pseudomonas* exotoxin A, inhibit protein synthesis at the ribosomes[28];

other intracellular toxins of gut pathogenic bacteria, such as heat-labile *E. coli* toxin, cause massive fluid and electrolyte movements in the small intestine, resulting in life-threatening diarrhea.[29]

Endotoxins (Figure 6.1) are lipopolysaccharides (LPSs) or lipooligosaccharides (LOSs), which are primarily found in the cell walls of bacteria within the Gram-negative phylum Proteobacteria. LPS binds to the LPS-binding protein (LBP) in serum, which transfers it to CD14 on the membrane of the host cell, where Toll-like receptor 4 (TLR4) (see Section 6.2.1.2) in mammals has been adapted primarily to recognize LPS.[30] A complex of TLR4 and the soluble protein myeloid differentiation factor 2 (MD-2), which is required for LPS signaling, is formed in the Golgi and transported to the cell surface or secreted as a soluble molecule.[31] Secretory plasma forms of CD14 may help LPS activation of CD14-deficient cells,[32] and certain forms of LPS may activate TLR4 independent of CD14.[33] Once LPS binds to TLR-4–MD-2, an early myeloid differentiation factor 88 (MyD88)–dependent response induces the early activation of nuclear factor κB (NFκB),[34] which leads to the production of inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1),[35] as well as a delayed MyD88-independent response, which induces interferon β.[36]

6.1.4 *Host–environment interaction influencing pathogenicity and research interference*

Apart from having many nonmicrobial side effects on animals, environmental stress may induce infectious disease; for example, in rats latently infected with *Mycoplasma pulmonis*, increased air concentrations of NH_3[37] or deficiency of vitamins A and E[38] may induce respiratory disease. Acidification[39] or chlorination[40] of the drinking water is known to reduce the incidence of clinical cases of *P. aeruginosa*-related problems in mice significantly. However, acidification of the drinking water changes the entire gut microbiota, and for nonobese diabetic (NOD) mice, this further increases the incidence of type 1 diabetes.[41] Proper ventilation of the animal unit is essential. Lowered air exchange may lead to respiratory disease caused by bacteria with low pathogenicity, such as *Staphylococcus xylosus*.[42] The experiment itself may be a stress factor. Postsurgical infections with bacteria such as *P. aeruginosa, E. coli*, and *S. aureus* are well known.[43] A special problem in laboratory animal medicine is imposed by experimental immunosuppression, which is a tool in some experiments. Various opportunistic members of the host microbiota, such as staphylococcal species,[44] may propagate and interfere with the model as a cause of immunosuppression.

6.2 Mechanisms of pathogenicity and research interference from the commensal gut microbiota

The impact of bacteria differs from that of viruses and parasites in the sense that all conventional and barrier-bred animals do harbor a range of different bacteria, while animals can be kept free of both viruses and parasites. To do so for bacteria, animals need to be reared in isolators. Bacterial impact on experiments is therefore far less controllable and definable than the impact caused by other types of microorganisms, such as viruses and parasites.

In the nineteenth century, it was discussed whether life without microbiota at all was possible[45,46]; today, there seems to be no doubt that the microbiota, sometimes referred to as the "normal flora," play an important role in mediating or preventing chronic inflammatory and metabolic processes in humans and animals.[47–49] Therefore, an understanding among laboratory animal scientists seems to be slowly evolving that the microbiota have an impact on animal models, and that this is relevant to consider in animal research to achieve reduction and refinement as well as higher predictive validity.[50] This fact has actually been clear through many years of work with germ-free animals, which exhibit some germ-free-associated characteristics (GACs) (i.e., non-microbiota-related traits present in germ-free animals), as well as some microflora-associated characteristics (MACs) (i.e., microbiota-related traits only present in animals with a microbiota).[51,52] However, differences in microbiota composition less dramatic than being germ free or not are also increasingly recognized as having an essential impact on the expression and variation in animal models.[50] This impact may be generated both through the animal immunity and through the animal metabolism; in many models, it is most likely to be a combination of the two. Although also within this field some specific bacterial species may be pointed out as especially important for certain models, their impact is based on their quantitative presence rather than their sole presence or absence, and some of them are symbionts rather than pathogens. Furthermore, many of these only exert their actions in conjunction with other members of the microbiota, and also with a range of other host and environmental factors.

6.2.1 Host–microbial interactions within immunity

6.2.1.1 Interactions within acquired immunity

Various parts of bacteria may trigger the immune system. An epitope is the antigenic determinant of a larger molecule specifically recognized by the acquired immune system, which will produce specific humoral

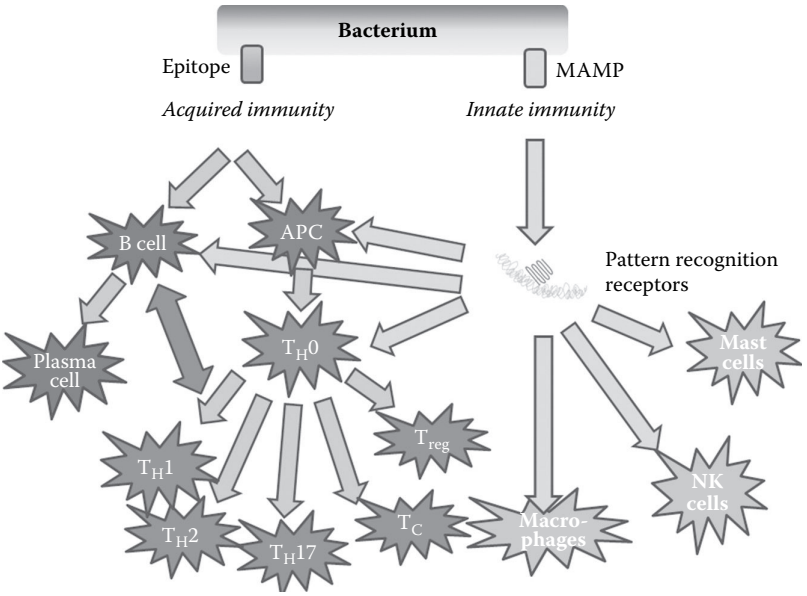


Figure 6.2 (See also color figure in the insert) Pathways for bacterial stimulation leading to humoral immunity (red cells), cellular immunity (blue cells; T, T cell; APC = antigen-presenting cell), or innate immunity (green cells; MAMP = microbial-associated molecular pattern; NK = natural killer cell).

or cellular antiepitope reactions (Figure 6.2). One molecule may express several epitopes, and as the bacterial wall consists of several molecules involved in both its structure and its functions, one bacterium may contain hundreds of epitopes, each of them inducing a specific immune reaction against them.

Naïve T cells (T_H0) of the host do not respond to epitopes by themselves but need them to be presented by antigen presenting cells (APCs) (Figure 6.2.) via carrier molecules such as MHC (major histocompatibility complex) and CD1.[53] Most host cells may act as nonprofessional APCs to stimulate double-positive $CD4^+CD8^+$ T_H0 via MHC class I molecules. By stimulation, T_H0 may turn into single-positive $CD4^+$ T-helper cells (T_H), that is, T_H1 protecting against intracellular bacteria, T_H2 mostly relevant against parasites, and T_H17 facing extracellular bacteria and fungi, as well as $CD8^+$ cytotoxic T cells (T_C). To stimulate these, both MHC classes I and II must be expressed by professional APCs, of which the most important are the dendritic cells (DCs), also sometimes designated the servers of the immune system because of their broad range of antigen presentation and their potency for activation of T_H1s.[54–56] Alternatively, T_H0 may become regulatory T cells (T_{reg}), which in the most common forms express the markers CD4, CD25, and Foxp3.[57] T_{reg}s suppress immune responses of

other cells, primarily to prevent overreaction to commonly encountered antigens, such as the members of the animal microbiota.[58] They direct the host response in a more innate direction, and they are important players in avoiding autoimmunity.[59,60] Other regulatory or suppressor T-cell populations are known, such as $CD4^-$ T_H3 cells, which are important in the induction of oral tolerance in the gut.[58]

Whether a T_H0 turns into a T_H or a T_{reg} depends on factors such as the magnitude of the stimulation, the age of the host, and the anatomic site. If T_H0s receive weak signals, they tend to become T_H2s rather than T_H1s and T_{reg}s rather than T_H17s.[61] In early life, there seems to be a window open during which it is easier to stimulate the formation of tolerance through T_{reg}s[62]; in the nose, it seems as if there is a more lifelong ability of inducing tolerance.[63] Especially in the gut, it is essential to avoid inflammatory disease later in life because that oral tolerance to the microbiota is established in the open window early in life.[64] In mice, the microbiota establishes itself around weaning, and this establishment to a certain extent primes the future immune responses of the host.[62] Variation in how laboratory animals meet their future microbiota in early life is therefore likely to cause variation in how they respond to model disease development later in life, as specific human diseases commonly modeled in rodents are driven by specific subsets of T cells.[50]

In the gut, the current stimulation of the dendritic cells from the microbiota induces IL-12 secretion, which will increase levels of T_H1 at the cost of T_H2.[65] Germ-free animals, therefore, have more T_H2s and fewer T_H1s.[66] Stimulation with bacterial polysaccharide A (PSA) (e.g., from *Bacillus fragilis*) can correct this.[67] The B cells present bacterial antigens for T cells, which signals back with cytokines to make the B cell turn into a plasma cell, which in the gut mucosa will produce immunoglobulin A, which will make the bacteria clump and thereby have a preventive impact on the microbiota's penetration potential.[68] Inflammatory bowel disease (IBD) occurs because of either a T_H1/T_H17 response (morbus Crohn) or a T_H2 response (ulcerative colitis) to the gut commensals[69]; therefore, under germ-free conditions[70–74] (and for some models even under barrier-protected conditions[47]), IBD models develop no disease at all, although IL-2-deficient mice do show mild focal intestinal inflammation.[75] Some bacterial species such as *E. coli, Enterococcus faecalis*, or *Bacteroides vulgatus* enhance disease,[76–78] and others such as *Lactobacillus* spp., *Bifidobacterium* spp., or *E. coli* Nissle seem to be protective against experimental IBD.[79–83]

The lack of *segmented filamentous bacteria* (*SFB*) (*Candidatus savagella*) in mice, as has been the case with mice from some commercial breeding centers, may reduce the T_H17 inflammatory response.[84,85] In mice[86] as well as in humans,[87] development of type 1 diabetes correlates to a low number of T_{reg}s and a strong reaction of T_H1 and T_H17; therefore, different microbial statuses have been reported to influence the immunology and to some

extent the diabetes incidence in mice.[88,89] In spontaneous rodent type 1 diabetes models, the fecal microbiota differs and contains an increased number of *Bacteroides* spp. prior to diabetes onset.[90] Treatment with the anti-gram-positive antibiotics, such as fusidic acid[91] and vancomycin,[92] reduces the incidence of type 1 diabetes in spontaneous rodent models, especially after early life treatment. In some atopic dermatitis mouse models, which in their chronic phase are dominated by a T_H2 response,[93,94] the cytokine levels in the hapten-treated ears correlate to the composition of the gut microbiota before induction.[95]

6.2.1.2 Interactions within innate immunity

The innate immune system is a designation for that part of the host defense that is nonspecific and has no memory. Invading bacteria are met by its various mechanisms and cells, and the acquired immune system may be recruited.

Essential parts of the bacteria are so-called microbial-associated molecular patterns (MAMPs), previously known as pathogen-associated molecular patterns (PAMPs). These are molecular structures in bacteria that will react with pattern-recognition receptors (PRRs) in the host (Figure 6.2). MAMPs, like epitopes, are parts of structural or functional molecules in the bacteria, and this feature is in no way limited to pathogenic bacteria. Among the most important PRRs are the toll-like receptors (TLRs). Different types of TLRs are present on a range of different cell types,[33,36,64,96–115] and they are stimulated by different MAMPs (Figure 6.3); depending on the combination, this may have both pro- and anti-inflammatory,[99] as well as cytometabolic, effects, such as when TLR2 signaling in the APCs has an impact on vitamin A metabolism, which leads to the creation of gut-homing T cells.[116] TLR2 and its coreceptors TLR1, TLR6, and TLR10 even occur both alone or in combination with TLR2, and interaction with the single receptors or one of the dimers leads to different cellular actions.[99] Some of them (i.e., at least TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9) can be directly stimulated by specific bacterial products[99]; others, such as TLR8, which primarily is stimulated by viruses, may be upregulated in cells after bacterial phagocytosis.[102] MAMPs of different types are dispersed and shared between members of the microbiota; therefore, the combinations of various innate responses that an animal host may have to a diverse microbiota seem endless. Also, the age of the animal makes a difference; for example, stimulation of TLR1, TLR2, and TLR4 in early life leads to higher production of IL-6 than stimulation later in life.[117] Inflammatory hypernociception induced by LPS or various cytokines is reduced in germ-free mice.[118]

Another important group of PRRs is the nucleotide-binding oligomerization domain-like (NLR) receptors, which are intracellular MAMP recognizers found in lymphocytes, macrophages, dendritic cells, and other

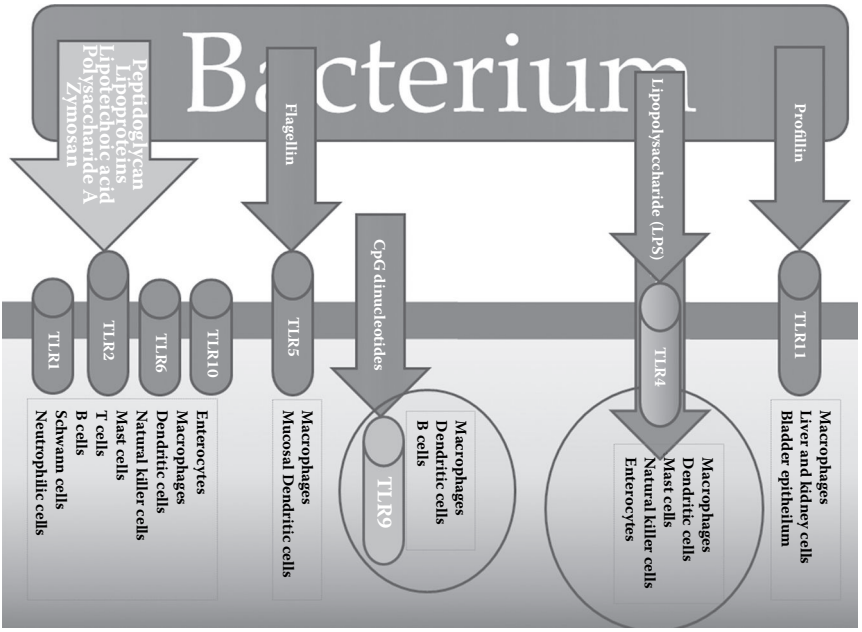


Figure 6.3 (See also color figure in the insert) Toll-like receptors (TLRs) interacting with bacteria in an animal host. Most bacteria-responding TLRs are membrane bound (green); others are endosomal (orange), and TLR4 is both (green/orange). Some are stimulated by compounds of Gram-positive bacteria (blue), others by compounds of Gram-negative bacteria (red), and some by compounds from both Gram-staining types (red/blue). They are expressed on a vast amount of different cell types, some examples of which are given.

types of cells, such as those of the epithelium.[119] As TLRs, they can activate NFκB to regulate inflammatory and apoptotic response; likewise, they can be stimulated by bacterial components (e.g., flagellin from *Salmonella, Legionella, Listeria*, or *Pseudomonas* is recognized by NLRc3, NLRc4, and NLRc5).[119] The host may also secrete compounds, such as the endogenic mannose-binding lectin (MBL),[120] which with their sugar structures have affinity for the sugar-binding adhesins of bacteria. MBL can activate the complement system by binding the C4b subunit and releasing C4a into the bloodstream, which activates the cascade leading to the membrane attack complex, which will destroy the bacterium.[121] Macrophages and dendritic cells possess a mannose-binding receptor with the same capability.[122]

Innate immune system cytokines, such as IL-1, IL-6, and TNF-α, induce "sickness behavior," which is believed to change the priorities of the organism to enhance recovery and survival[123] and may very well originate from an innate gut microbiota provocation, as stressing animal models changes their microbiota.[51] The composition of the gut microbiota,

maybe especially Firmicutes member levels, has an impact on responses in stress tests.[124,125]

The gut microbiota stimulates Paneth cells[126] or enterocytes[127] to produce various antimicrobial peptides, which will regulate the microbiota composition.

6.2.2 Host–microbial interactions within metabolism

Especially within animal models of the metabolic syndrome, there seems to be an association between the animal microbiota and some of the metabolic parameters. In leptin-deficient obese mice, there is a strong correlation between glucose levels and the composition of the gut microbiota.[128] In C57BL/6 mice, long-term oral ampicillin improves glucose tolerance.[129] However, this effect is mainly because of an early life impact on glucose tolerance, and the effect ceases immediately after termination of treatment; hereafter, the glucose tolerance may even decrease.[130] Obesity seems to be associated with changes in the number of Bacteroidetes and Firmicutes,[131,132] with increased levels of Prevotellaceae in C57BL/6 mice linked to decreased glucose tolerance, while Lachnospiraceae seem quantitatively correlated to increased glucose tolerance.[128] Germ-free mice are 40% leaner because of less body fat, although they eat 30% more compared to conventional mice,[133] and colonization of them with a microbiota from obese mice compared to one from lean mice increases total body fat.[131] A number of pathways for this have been proposed, as reviewed by Cani et al.[134]; for instance, (a) bacterial colonization promotes intestinal monosaccharide absorption through a doubling of the density of capillaries in the small intestinal villus epithelium[135]; (b) microorganisms extract energy from undigested food components by fermenting it into short-chain fatty acids (SCFAs), which in addition to other effects may act as signals for at least two G-protein-coupled receptors, GPR41 and GPR43, essential for the development of obesity[136,137]; and (c) bacteria may participate in hepatic lipogenesis because of their expression of several key enzymes, such as acetyl-coenzyme A (CoA) carboxylase and fatty acid synthase,[133,138] and suppress fatty acid oxidation in the liver and in skeletal muscle because of their expression of adenosine monophosphate (AMP)-activated protein kinase.[139]

Excess dietary fat increases plasma LPS levels, a phenomenon known as metabolic endotoxemia,[140] and this is linked to specific changes in the gut microbiota with a marked reduction in *Bifidobacterium* spp., *Bacteroides*-related bacteria, and *Eubacterium rectale/Clostridium coccoides*.[141] Mice knocked out for CD14 or TLR4 have decreased sensitivity to the development of diabetes,[142] obesity,[143] and atherosclerosis.[144] Acylglycerols from the endocannabinoid system, a group of neuromodulatory compounds and

receptors for these in the brain, may reduce metabolic endotoxemia and systemic inflammation.[145,146]

Prebiotics (i.e., nondigestible carbohydrates known as oligosaccharides) in dietary intake act by their growth-promoting effect on specific microbiota members, such as *Bifidobacterium* spp.[147] and *Faecalibacterium prausnitzii*,[148] as well as their affinity for the sugar-binding adhesins for unhealthy bacteria.[149] They reduce hunger and circulating ghrelin, thereby decreasing total energy intake and body weight gain by about 10%[150–152]; increase plasma levels of glucagon-like peptide 1 (GLP-1)[150,151,153] and peptide-YY (a short appetite-reducing peptide released by cells in the ileum and colon in response to feeding)[151]; and decrease interferon (IFN) γ and IL1-β.[147] Host–bacterial interactions seem to control gut barrier function as treating leptin-deficient obese mice with prebiotics reduces the permeability of their gut, as well as the LPS concentrations in the blood and cytokine concentrations in plasma; this effect seems to be driven through GLP-2.[154]

In general, vitamins need to be supplemented to animals, although food-related lactic acid bacteria and *Bifidobacteria* can de novo synthesize and supply vitamins, such as the B vitamin folate, which requires both 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and para-aminobenzoic acid (pABA),[155] which can be shown by increased folate levels in rats supplied with *Bifidobacteria* with these properties.[156] However, vitamin deficiencies in germ-free animals, for example, are more likely to be related to decontamination of diets rather than the lack of a microbiota.

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chapter seven

Systematic classification of bacteria

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7.1 Domains of life

In 1977, Woese and Fox published their classic paper dividing the "phylogenetic tree of life" into three domains (then termed *kingdoms*)—Bacteria, Archaea, and Eucarya[1,2]—a division that still holds today. The three domains can be further divided into kingdoms, phyla, classes, orders, families, genera, species, and subspecies. Within microbiology, different species/subspecies can then be further divided into, for example, serotypes, as done for many pathogens like *Salmonella*, and strain level (Table 7.1).[3,4] Noncellular life, such as viruses and phages (virus-attacking bacteria) are not included in the three domains of life, but with the discovery of megaviruses with a genome size equivalent to many bacteria, it has been argued that viruses form a fourth domain of "life."[5] Whatever their status—as a fourth domain of life or not—there is no doubt that viruses play important roles in shaping the microbiome of rodents.[6,7]

7.1.1 Operational taxonomic units

The term *operational taxonomic unit* (OTU) is another central element in both numerical taxonomy (based on phenotypic data), for which an OTU typically represents an individual strain or group of isolates with similar phenotypic properties, and high-throughput sequencing-based studies, for which organisms are not directly observed. Here, an OTU is used to delineate groups of sequences/reads above a given similarity threshold. Typically, a similarity level of 97% is used for delineating sequences at the species level when based on the 16S ribosomal RNA (rRNA) gene. This similarity value is a reasonable "working value," but it is important to

Table 7.1 Taxonomic Position of the Probiotic Strain *Bifidobacterium animalis* subsp. *lactis* BB-12

Level	Example
Domain	Bacteria
Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Bifidobacteriales
Family	Bifidobacteriaceae
Genus	*Bifidobacterium*
Species	*Bifidobacterium animalis*
Subspecies	*Bifidobacterium animalis* subsp. *lactis*
Strain	*Bifidobacterium animalis* subsp. *lactis* BB-12

bear in mind that many validly described bacterial species differ by much less than 3%.[4,8,9]

7.1.2 Members of the mice and rat microbiome

In mice and rats, as well as in humans, Bacteriodetes and Firmicutes are the most abundant phyla, in general accounting for 50–90% of the total prokaryotic gut microbiota. Other members of the core gut microbiome of mice and rats are Actinobacteria, Deferribacteres, Proteobacteria, Tenericutes, Verrucomicrobia, and the candidate phylum TM7 (Table 7.2). Rats tend to have a much more diverse gut microbiota than mice and humans, with two to three times as many OTUs detected.[10,11] This should be kept in mind when planning studies involving gut microbiota characterization based on, for example, 16S rRNA gene amplicon sequencing, as the rat fecal microbiota in many cases has to be sequenced deeper to reach adequate coverage. Unicellular eukaryotes (yeast, fungi, blastocytes), Archea, and phages are all members of the rodent microbiota. However, they are ignored in the majority of studies, where focus is solely on the Bacteria. However, recent publications showing the involvement of unicellular eukaryotes in disease models (as well as human health and disease) and the influence of phages on microbiome composition has led to greater interest in these organisms.[7,12–14]

Table 7.2 Overview of the Systematics of the Important Phyla within Laboratory Animal Bacteriology with Some Relevant Species Given as Examples

Kingdom	Phylum	Class	Order	Family	Genus	Species (example)
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Streptococcus*	*S. zooepidemicus*
				Enterococcaceae	*Enterococcus*	*E. faecium*
				Lactobacillaceae	*Lactobacillus*	*L. murinus*
			Bacillales	Staphylococcaceae	*Staphylococcus*	*S. aureus*
				Bacillaceae	*Bacillus*	*B. anthracis*
				Listeriaceae	*Listeria*	*L. monocytogenes*
		Erysipelotrichi	Erysipelotrichales	Erysipelotrichidae	*Erysipelothrix*	*E. rhusiopathiae*
		Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*C. piliforme*
	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Micrococcus*	*M. luteus*
				Actinomycetaceae	*Actinomyces*	*A. bovis*
				Mycobacteriaceae	*Mycobacterium*	*M. lepraemurium*
				Corynebacteriaceae	*Corynebacterium*	*C. kutscheri*
			Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	*B. animalis*
	TM7	Candidate phylum only detected by sequencing				
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Salmonella*	*S. typhimurium*
					Escherichia	*E. coli*
					Citrobacter	*C. rodentium*
			Pasteurellales	Pasteurellaceae	*Pasteurella*	*P. pneumotropica*
					Haemophilus	*H. influenza murium*
					Actinobacillus	*A. muris*

continued

Table 7.2 (continued) Overview of the Systematics of the Important Phyla within Laboratory Animal Bacteriology with Some Relevant Species Given as Examples

Kingdom	Phylum	Class	Order	Family	Genus	Species (example)
			Pseudomonales	Pseudomonadaceae	*Pseudomonas*	*P. aeruginosa*
		Betaproteobacteria	Burkholderiales	Alkaligenaceae	*Bordetella*	*B. bronchiseptica*
				Spirillaceae	*Spirillum*	*S. minus*
		Epsilonproteobacteria	Campylobacterales	Helicobacteriaceae	*Helicobacter*	*H. hepaticus*
				Campylobacteriaceae	*Campylobacter*	*C. coli*
	Bacteroidetes	Bacteroidea	Bacteroidales	Bacteroidaceae	*Bacteroides*	*B. fragilis*
				Prevotellaceae	*Prevotella*	*P. copri*
				Rikenellaceae	*Alistipes*	*Alistipes* spp.
					Rikenella	*Rikenella* spp.
				S24-7	Not further characterized	
		Sphingobacteria	Sphingobacteriales			CAR bacillus (rodent)
	Verruccomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	*A. muciniphila*
	Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	*Fusobacterium*	*F. necrophorum*
				Leptotrichiaceae	*Streptobacillus*	*S. moniliformis*
	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum*	*M. schaedleri*
	Spirochaetae	Spirochaetes	Spirochaetales	Spirochaetaceae	*Treponema*	*T. paraluis cuniculi*
				Leptospiraceae	*Leptospira*	*L. interrogans*
	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	*Mycoplasma*	*M. pulmonis*
			Acholeplasmatales	Acholeplasmataceae	*Acholeplasma*	*A. cavigenitalium*

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chapter eight

Firmicutes

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8.1 Introduction

Most Firmicutes are Gram positive, although some that are not relevant here stain Gram negative. They are defined by having a low guanine-cytosine content compared to the other Gram-positive phylum Actinobacteria. The phylum contains both rods and cocci, and some produce endospores. They form round cells, called cocci (singular coccus), or rod-like forms (bacillus).

Cultivable sporogenic rods have traditionally been divided into *Bacillus*, which are aerobic or facultatively anaerobic, and *Clostridium*, which are strictly anaerobic. By large this division still holds, though several genera such as Lysinibacillus have been separated from the Bacillus genus during recent years. *Bacillus* spp. are catalase positive. One *Clostridium* species, *C. piliforme*, is one of the most important bacteria in laboratory animal bacteriology, but it cannot be cultivated by traditional techniques, the same as for a range of other *Clostridium* spp.

Leptin-deficient obese mice have significantly more Firmicutes and fewer Bacteriodetes species compared to their wild-type and heterozygous littermates.[1] In stressed mice, there seems to be a correlation between their Firmicutes levels and their responses in stress tests.[2] Mice that by knockout are made unable to produce the active form of vitamin D or the vitamin D receptor have fewer Firmicutes bacteria in their feces compared with wild-type mice.[3] On the other hand, the families Lactobacillaceae and Lachnospiraceae show increased abundance in feces from these knockout mice.[3]

8.2 Streptococcaceae

Streptococcaceae are catalase negative, grow pinpoint colonies, and are grouped in chains (see Figure 3.2, item 2) or in pairs (see Figure 3.2, item 3). Streptococcaceae may be divided into the following genera: *Streptococcus, Aerococcus,* and *Gemella*, which are more or less important in laboratory animal bacteriology; and *Pediococcus* and *Leuconostoc*, which are unimportant for laboratory animals. The basis for differentiation between genera is given in Table 8.1. All media for cultivation of Streptococcaceae must be enriched, that is, they should contain serum or the equivalent.

Streptococci are catalase negative, microaerophilic, grouped in chains (see Figure 3.2, item 2), and form pinpoint colonies on most agars.

Table 8.1 Differentiation of Different Genera of Gram-Positive Cocci that Can Be Isolated from Laboratory Rodents and Rabbits under Aerobic or Microaerophilic Conditions

	Micrococcus[a]	Staphylococcus	Aerococcus	Streptococcus	Enterococcus	Gemella
Aerobic growth	+	+	+	+	+	+
Anaerobic growth	–	+	+	+	+	+
Microaerophilic	–	–	–	+	+	–
OF (oxidative-fermentative) test	O	F	F	F	F	F
Microscopic appearance	Pair/tetrades/grapes	Grapes	Pairs/tetrades	Chains	Chains	Single/pairs
Catalase	+	+	Weak or –	–	–	–
Colony pigmentation	+	+	–	–	–	–
Colony size > 1 mm	+	+	–	–	–	–
Lysostaphin sensitivity	–	+	–	–	–	–
Growth in						
6.5% NaCl	d	+	+	–	+	–
12% NaCl	–	+	–	–	–	–

[a] Actinobacteria.
F = fermentative; O = oxidative.

8.2.1 Impact on the host

Streptococcus spp. of Lancefield's groups A, B, C, and G may be found in laboratory animals all over the world. Group A is uncommon in barrier-housed colonies; the other types are more common,[4,5] although many colonies may be free of these bacteria, especially hemolytic types. The observed prevalences of a certain species within infected barrier-bred colonies are generally 8% to 10%. *Streptococcus pneumoniae* is most common in guinea pigs, less common in rats and rabbits, and rare in mice.[6,7] The most common type in guinea pigs is type 19; types 2, 3, 8, 16, and 19 have been reported in rats. The prevalence within infected colonies may vary from 15% to 55%. Most streptococci, *S. pneumoniae* included, may spread from humans to animals and vice versa. Infection is mostly by droplets through the intranasal route. In barrier-maintained colonies of laboratory animals, streptococci are mainly of human origin.

Most spontaneous infections with streptococcal species are nonclinical. It is generally considered of major importance to differentiate between β-hemolytic streptococci (i.e., those that on blood agar form a clear zone around the colony) and the other types (i.e., those that are either nonhemolytic or α-hemolytic, forming a green zone around the colony). This differentiation is based on most pathogenic streptococci being β-hemolytic, although some of the other types also may show some pathogenicity. According to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines for health monitoring[8] (see Chapter 1), infections with streptococci other than *S. pneumoniae* in rodent colonies are only to be reported if these streptococci are β-hemolytic.

8.2.1.1 *Lancefield's groups A, B, C, and G*

Clinical expressions in rodents have mostly been provoked by experimental infections with β-hemolytic streptococci.[9,10] *Streptococcus equi* subsp. *zooepidemicus* (group C) is the cause of various pyogenic processes—such as lymphadenitis, submandibular abscess, pneumonia, pleuritis, pericarditis, peritonitis, arthritis, conjunctivitis, opthalmia, mastitis, otitis media, and otitis interna—resulting in a high mortality rate in guinea pigs.[11] Typical species in laboratory rats and mice are group G, typically *S. dysgalactiae equisimilis,*[4] which may be the cause of milder symptoms, such as conjunctivitis, and group B, such as *S. agalactiae,*[4] which may cause abscesses and eventually mortality in newborn rats.[12] Group A streptococci in rats and mice cause disease equivalent to that caused by group C streptococci in guinea pigs.

8.2.1.2 Streptococcus pneumoniae

Disease caused by *S. pneumoniae* seldom occurs in mice, and if it does, it is mostly related to stress caused by a poor environment in the animal

unit or nutritional deficiencies. The innate immunity toward the organism is impaired in old mice.[13] In rats, the agent causes lung disease[7] with additional symptoms from conjunctiva and the ears. The first symptom may be a mucopurulent discharge from the nose, but later the disease progresses and noisy, abdominal respiration occurs. Pathological changes are dominated by fibrin with various grades of focal bronchopneumonia developing into lobar fibrinous pneumonia. In rare cases, liver, spleen, kidney, and testes may be involved. In guinea pigs, similar symptoms may be present, but often unexpected deaths are the only visible signs of the infection. In rabbits, disease often quickly leads to septicemia after signs of dyspnea and depression.

8.2.1.3 Lancefield's group D

Most enteric streptococci have been reclassified as *Enterococcus* spp.,[14] but high-throughput sequencing based on investigations of the gut microbiota of rodents does reveal *Streptococcus* spp. as parts of the microbiota.[15] If isolated, these are likely to be diagnosed as belonging to the *S. bovis* group, which expresses the group D antigen and probably plays a role for the gut permeability of the host.[16]

8.2.1.4 Lactococcus *spp.*

Lactococci appear in high abundance in high-throughput sequencing-based characterization of both cecal contents and feces from mice,[17] and *Lactococcus lactis,* which is frequently isolated from rodents, has been related to increased incidence of type 1 diabetes in nonobese diabetic (NOD) mice,[18] although feeding these bacteria to the mice did not seem to have a major impact on the incidence.[19]

8.2.1.5 Gemella

Two species of *Gemella* are of some importance: *G. haemolysans*, which is β-hemolytic, and the nonhemolytic *G. morbillorum,* previously known as *Streptococcus morbillorum. Gemella* spp. are found in laboratory rodents in using both cultivation and sequencing-based methods,[17] but their impact on rodents is not clear.

8.2.2 Characteristics of the agent

Streptococci grow as small, circular, transparent, so-called pinpoint colonies on blood agar after 24 to 48 h. α-Hemolysis is the most common; β-hemolysis occurs; γ-hemolysis is rare. *Streptococcus pneumoniae* appear tiny, smooth, and flat with a small rim of α-hemolysis after 24 h of incubation on blood agar. After 24 h, the colonies develop a characteristic ring-like appearance with a raised periphery around a depressed center. In the microscope, the cocci are found to be not as round as staphyloccci. Most

streptococci occur in chains (see Figure 3.2, item 2); *S. pneumoniae*, which are lancet shaped, are arranged in pairs (see Figure 3.2, item 3). *Gemella,* in their cultural and morphological characteristics, resemble streptococci; that is, they are catalase negative and form pinpoint colonies. However, although Gram positive, they are easily destained in the Gram staining and may therefore falsely be characterized as Gram negative in the initial part of the identification process.

Cultivation may be obtained after direct smear on blood or chocolate agar. Incubation should be 24 to 48 h at 37°C. Most streptococci will grow in an aerobic environment, but cultivation is facilitated by 5% carbon dioxide (microaerophilic incubation). Columbia blood agar is superior to ordinary blood agar. If routine sampling is performed from healthy animals, it should be noticed that streptococci are often overgrown by faster-growing organisms. For propagation, various broths are available (e.g., soy broth).

The sampling site of choice for cultivation from healthy animals is the nasopharynx sampled through the trachea (see Chapter 2). The nose and the genital organs may be useful as well. For *S. pneumonia*, saline washing of the tympanic cavity may be useful, especially in rats. The enteric *Streptococcus* and *Enterococcus* spp. are most likely to be identified in feces.[17]

Streptococci may be grouped within Lancefield's groups. In laboratory animal bacteriology, antibodies against types A, B, C, D (enterococci), F, and G are of common use, but several laboratory animal streptococci of less importance are of other types. Commercial kits for Lancefield's groups are available, such as SLIDEX® from bioMérieux (France) and the StrepPRO™ Grouping Kit from Hardy Diagnostics (USA). Latex agglutination kits for the identification of *S. pneumoniae* are available from the same suppliers. Identification of *S. pneumoniae* in such kits should be regarded as presumptive and should be confirmed by additional means.

Eighty-four different types of *S. pneumoniae* have been identified based on differences in capsular polysaccharide antigens. These may be differentiated by the Neufeld reaction (i.e., visualizing the capsule, which swells when the isolate is mixed with an anticapsular serum).[8]

Streptococci may be identified by their biochemical reactions, as shown in Table 8.2. The most important test for identification of *S. pneumoniae* is the test for sensitivity to optochin, as *S. pneumoniae* produce an autolysin in the presence of surface-active agents, such as bile salts or optochin. The optochin test is also commercially available as disks to place on an agar. A 15-mg disk is placed on a blood agar plate, and *S. pneumoniae* species will demonstrate a sensitivity zone with a diameter of at least 10 mm. Alternatively, the bile salt sensitivity test may be used. One drop of phenol red is added to 1 ml of a heavy broth culture, and NaOH is added until the color turns orange-red. Then, two or three drops of sodium taurocholate are added, which after 5 min dissolves the bacteria—if these are bile salt

Table 8.2 Identification of Streptococcaceae and Enterococcaceae Found in Rodents and Rabbits after Grouping with Lancefield's Antigens

Group A		
S. pyogenes		
Group B		
S. agalactiae		
Group C	**Hemolysis**	**Trehalose**
S. dysgalactiae	α	+
S. equisimilis	β	+
S. zooepidemicus	β	–
Group D	**Pyruvate**	**Arabinose**
Enterococcus faecalis	+	–
E. faecium	–	+
Group G		
No further identification		

sensitive. The API 20 Strep kit from bioMérieux identifies most laboratory animal streptococci in 4 or 24 h. An alternative is to identify isolates by polymerase chain reaction (PCR) or sequencing, which normally fits well with results achieved by serotyping.[20]

8.3 Enterococcaceae

Enterococci could most easily be described as enteric streptococci; that is, they are catalase negative and microaerophilic; they form pinpoint colonies and are grouped in chains. Most of them react with Lancefield's antibodies group D and may be divided into two groups: those that are resistant to tellurite and those that are not. *Enterococcus faecalis* will grow on tellurite-containing agars, on which it forms large, black colonies. This is routinely used as a selective and indicative method for isolation of enterococci. However, the most common species in small rodents is *E. faecium*, which will not grow in such media. Therefore, this system should not be used for isolation of enterococci in laboratory animal bacteriology. The prevalence of enterococci in rodents ranges between 15% and 40%. In rodents, enterococci have generally been considered opportunistic or even symbiotic; that is, they are seen as having a positive impact on the intestinal function of the host. However, in a more modern understanding, they are considered facultative pathogens,[21] and *E. faecalis* enhance inflammatory bowel disease (IBD) in interleukin (IL) 10 knockout mice,[22,23]

which is probably linked to its production of gelatinase.[24] Principles for identification are similar to what has been described for streptococci, and identification may be performed according to Table 8.2 Alternatively, the commercial kit API 20 Strep (bioMérieux) or PCR/sequencing-based methods may be applied.

8.4 *Aerococcaceae*

Aerococci are grouped in pairs or tetrades and therefore may be taken as micrococci at microscopy. *Aerococcus viridans* is occasionally isolated from the respiratory system of both rodents and rabbits, but any important impact on the animal or its use as a model has not been described. It forms pinpoint colonies on blood agar after 24 h of microaerophilic incubation, with an evident greening in the agar around the colony, especially after longer incubation. Identification may be performed according to Table 8.1 or with the commercial kit API 20 Strep (bioMérieux).

8.5 *Lactobacillaceae*

Lactobacilli are Gram-positive rods. They grow on enriched media (e.g., blood or chocolate agar), but often food microbiology laboratories will use more sophisticated media, including yeast extract, tomato juice, de Man, Rogosa, and Sharpe (MRS) media, and so on. They form pinpoint colonies and—under aerobic conditions—a greening of the agar will be observed on blood agar. Microaerophilic cultivation is highly recommended. They are common inhabitants of the gut of laboratory rodents.[17] The altered Schaedler flora contain ASF 360, which cluster with *Lactobacillus acidophilus* and *Lactobacillus lactis*, and ASF 361, which is close to identical with *Lactobacillus murinus* and *Lactobacillus animalis*.[25]

Lactobacilli are often difficult to cultivate from the gut because of overgrowth from other organisms and are therefore by cultivation more frequently isolated from the respiratory or genital organs. However, in high-throughput sequencing-based studies of the gut microbiota, they often constitute a significant fraction.[17] The API 50CH and partly the API 20 Strep and the API 20A kits (bioMérieux) will identify lactobacilli to a species level, but full identification requires laboratories that are more specialized. A high level of *Lactobacillus* spp. is strongly correlated with low levels of inflammation in mice[26] and leptin in rats,[27] which also fits well with these bacteria acting protectively against IBD in IL-10 knockout mice,[28–31] allergic sensitization in mice,[32] and myocardial infarction in rats.[27] Ingestion of *Lactobacillus rhamnosus* in mice regulates emotional behavior and central γ-aminobutyric acid (GABA) receptor expression via the vagus nerve.[33] *Pediococcus* spp., which are classified within

Lactobacillaceae and are found in the gut of rodents, may by cultivation be difficult to distinguish from *Lactococcus* spp.

8.6 Leuconostaceae

Leuconostaceae are Gram positive, not spore forming, round or elongated in shape, anaerobic or aerotolerant, and commonly observed in the rodent gut.[17]

8.7 Staphylococcaceae

8.7.1 Impact on the host

Staphylococci may be found worldwide and in all species of animals. Some staphylococci are more host specific than others; however, in general, spread between species, including animal to humans and vice versa, should be expected. The majority of humans and animals are carriers of some sort of staphylococci. The major pathogen among these bacteria, *Staphylococcus aureus*, is typically found in two-thirds of the human population and is also found in many colonies of laboratory rodents.[34–36] However, as it is extremely rare in wild rodents,[37] laboratory rodents probably achieve them from close contact with humans. Other types of *Staphylococcus* common in laboratory rats and mice include *S. haemolyticus, S. xylosus, S. sciuri,* and *S. cohnii*.[35] The bacteria may be transmitted among hosts in various direct or indirect ways, including passive carriers among animal technicians.

Staphylococcal disease in immune-competent animals is mainly secondary—caused by trauma, stress, or the equivalent—and is characterized by pyogenic processes, such as abscesses in bite or surgical wounds, pneumonia in rodents kept in poorly ventilated units, and dermatitis in gerbils kept in too humid bedding. In immune-deficient animals, *S. aureus* may be a primary disease-causing agent (e.g., in the nude mouse, in which it causes multiple abscesses). Most pathogenic staphylococci—the most important of which is *S. aureus*—are coagulase positive, but coagulase-negative staphylococci also may cause disease in laboratory animals; for example, *S. xylosus* is known to cause intestinal disease in mice,[38] dermatitis in gerbils,[39] and pneumonia in immunosuppressed rats.[40] Interference with research is caused by secondary factors, such as stress or immunosuppression, but the presence of abscesses in immune-deficient animals, typically nude mice, also may be hazardous to research.

8.7.2 Characteristics of the agent

After 24 to 48 h on blood agar, staphylococci grow as pigmented colonies at least 1 mm in size on e.g. blood agar. After longer incubation, some species

Table 8.3 Characteristics of Staphylococcal Hemolysins

Hemolysin	Characteristics of hemolysis observed on blood agar	Erythrocytes hemolyzed		
		Sheep	Rabbit	Horse
α	Wide, sharply demarcated, clear zone	++	+++	–
β	Wide, sharply demarcated, dark zone	+++	+	–
γ	No hemolysis shown on blood agar, should be tested in an erythrocyte broth suspension	+++	+++	–
δ	Narrow, sharply demarcated, clear zone	++	++	+++

may show colonies more than 5 mm in size. At least four different hemolysins exist: α, β, γ, and δ. It should be noted that the type of hemolysis caused by these different hemolysins (Table 8.3) differs, for example, from streptococcal hemolysins named by equivalent Greek letters. Strains of animal origin produce β-hemolysin more frequently than the other types. In the microscope, staphylococci are spherical and are often grouped as grapes (see Figure 3.2, item 1), although some types (e.g., *S. xylosus*) are not so spherical and do not have a grape-like appearance.

Staphylococci grow easily after direct inoculation on blood agar. If the samples are heavily contaminated, a selective agar, such as mannitol salt agar (see Table C.11), may be used for inoculation. If isolation of *S. aureus* is the sole aim, Baird Parker agar (see Table C.10) is of great use.

From the diseased animals, the organ with the pyogenic process is sampled directly. From healthy animals, staphylococci may be isolated from all parts of the respiratory system, the skin, and the genitals.

The coagulase test is the first step in the identification process. The vast majority of coagulase-positive staphylococci in laboratory rodents are *S. aureus*. A simple way to identify *S. aureus* is by latex agglutination, such as by the SLIDEX Staph Plus (bioMérieux), although a few percent of the isolates will test as false negative.[41] If further identification is needed, staphylococci may easily be divided on the basis of simple biochemical characteristics (e.g., fermentation assays) and identified according to Table 8.4. Some strains of *S. xylosus*, which are pathogenic in rats, are arginine dehydrolysis positive and urease negative, which is opposite to typical strains of *S. xylosus*.[40] A commercial kit, API STAPH, is available from bioMérieux. Phage typing is a technique especially used for characterization of *S. aureus*. The isolate is incubated with a range of suspensions, each containing one of a number of well-characterized bacteriophages. Each type of *S. aureus* is lysed by certain bacteriophages and not by others. PCR and mass spectrometry are also widely used for identification of *S. aureus*.[42]

Table 8.4 Biochemical Reactions of Staphylococci Found in Rodents and Rabbits

	S. aureus	*S. cohnii*	*S. haemolyticus*	*S. hominis*	*S. lugdunensis*	*S. saphrophyticus*	*S. sciuri*	*S. simulans*	*S. xylosus*
Coagulase	+	–	–	–	–	–	–	–	–
Clumping factor	+	–	–	–	–	–	–	–	–
Acid from cellobiose	–	–	–	–	–	–	+	–	–
Lactose	+	–	d	+	–	+	–	+	+
Maltose	+	+	+	+	+	+	+	–	+
Mannitol	+	+	+	–	–	+	+	+	+
Mannose	+	–	–	–	+	–	d	d	+
Sucrose	+	–	+	+	+	+	+	+	+
Xylose	–	–	–	–	–	–	–	–	+
Arginine dihydrolasis	+	–	+	+	–	d	–	+	+/–[a]
Urease	d	d	–	+	d	+	–	+	+/–[a]

[a] Some strains of *S. xylosus*, which are pathogenic in rats, are arginine dihydrolasis positive and urease negative, which are opposite to typical strains of *S. xylosus*.
d = differs between strains.

8.8 Bacillaceae

8.8.1 Impact on the host

Only one species, *Bacillus anthracis*, the causative agent of anthrax, is known to be pathogenic in laboratory animals. Anthrax is primarily a disease of agricultural animals and humans; it causes acute septicemia. Mice are susceptible to infection with *B. anthracis* and the subsequent development of anthrax.[43] However, it is unlikely that this should become a major problem in mouse colonies because of worldwide programs to prevent anthrax in other animals and humans. *Bacillus* spp. of various kinds are common inhabitants of laboratory animals,[17] although the animals may not necessarily be infected or associated with these germs in the traditional sense. Spores often enter the animal facilities with the diets, especially if these are not or only partially autoclaved. The spores may then be found in the coat or in the digestive system. When applying DNA-based methods for identification, such spore-forming species may appear even in germ-free animals as their inactivated DNA is entering the gut when feeding the animals an irradiated diet.

8.8.2 Characteristics of the agent

In traditional bacteriological systematics, *Bacillus* spp. are characterized as Gram positive. However, in reality, some species are more or less Gram negative, while some species are Gram positive in young cultures and Gram negative in older cultures. The ability to form spores is therefore far more important in the identification process than the Gram stainability. The cells are typical rods, the size of which normally is much larger than rods of other genera. The sides are normally parallel, and some species (e.g., *Bacillus cereus*) form chains. Some species form rather characteristic colonies, which are large (2 to 4 mm), rough, and fatty, but all types of colonies are observed within this genus; therefore, identification of the genus cannot be based on colony morphology. *Bacillus* spp. all grow easily on simple, nonenriched media. Psychotropic species, with temperature optimum between 20°C and 25°C; mesophilic, with optimum around 30°C; and thermophilic, with optimum between 50° and 55°C, exist. Sporulation is best observed after incubation on a sporulation medium (see Table C.8).

The cecum and the trachea are the two most common sites of isolation.

The identification process should always start by characterizing the spores according to Figure 8.1. The easiest way, then, to identify *Bacillus* spp. is through the use of the combination of commercial kits API 50CH and API 20E (bioMérieux), a method primarily based on carbohydrate assimilation. Alternatively, Table 8.5 can be used. *Bacillus anthracis* is closely related to *B. cereus*. Differentiation between these two is, of course, essential, as the latter is extremely common. First, sensitivity to penicillin is applicable,

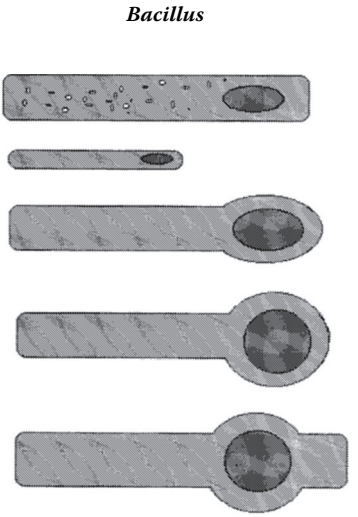


Figure 8.1 Characterization of *Bacillus* spp. according to the spore position, swelling around the spore, and the presence of metachromatic granules in the cytoplasm and characterization of *Clostridium* spp. according to the shape and position of the spores.

Table 8.5 Differentiation of *Bacillus* spp.

	Spore type	Motility	Anaerobic growth	Glucose gas	Starch hydrolysis	Voges-Proskauer
B. megaterium	1A	d	–	–	+	–
B. thuringiensis	1A	d	–	–	+	–
B. cereus	1A	+	+	–	+	+
B. mycoides	1A	–	+	–	+	+
B. anthracis	1A	–	+	–	+	+
B. licheniformis	1B	+	+	+	+	+
B. subtilis	1B	+	–	–	+	+
B. pumilus	1B	+	–	–	–	+
B. coagulans	2	+	+	–	+	+
B. polymyxa	2	+	+	+	+	+
B. macerans	2	+	+	+	+	–
B. circulans	2	d	d	–	+	–
B. sphaericus	3	+	–	–	–	–

d = differs between strains.

as *B. anthracis* is sensitive, while all *B. cereus*, except for type 1A, are resistant. *Bacillus anthracis* is nonhemolytic and thereby differs from the hemolytic *Bacillus mycoides*. Finally, *B. cereus* is motile; *B. anthracis* is not. Various PCR-based methods may also be applied for identification, such as sequencing of, for example, the gyrA and gyrB genes that often is very useful.[44–46]

8.9 Listeriaceae

8.9.1 Impact on the host

A range of species has been described within the genus *Listeria*, but only one species, *L. monocytogenes*, is known to cause disease in laboratory animals; however, this infection is rather uncommon. It may occur in rare cases in rabbits and guinea pigs; rats are rather resistant to infection. Although this agent traditionally is known to be pathogenic in mice, it does not play a major role as a spontaneous infection in laboratory mice. The bacterial epidemiological cycle is not fully known. Excretion is thought to be from the mouth, nose, and vagina. It may be found in humans, as well as in various parts of the environment, such as feed and water. According to the FELASA guidelines for health monitoring[47] (see Chapter 2), monitoring for *Listeria* infections is not mandatory. Most infections are subclinical, and disease is related mainly to either contaminated diets or immunodeficiencies of the host. In the latent infected carrier, the agent is found within the macrophages. Septicemia, which in rabbits may be peracute, may lead to death, after which blood and other fluids accumulate in the abdomen, thorax, and the pericardial sac; hemorrhages and lymph node edemas are also observed during necropsy. In more prolonged cases, various grades of depression and weight loss may be observed, and during necropsy, gray pinpoint necroses may be found in the liver, spleen, and uterus. Because of the last, abortions may be caused by infection with *L. monocyctogenes.* Meningitis and encephalitis may also be found. Bacterial excretion may be from the digestive, respiratory, or genital systems. Types of research interference other than those related to disease, death, or pathological changes have not been described.

8.9.2 Characteristics of the agent

Listeria is a short, rather thick rod, but especially in young cultures, coccoid forms may be observed. The bacterium is Gram positive, although destaining may occur in older cultures. *Listeria monocytogenes* is motile at 22°C, but not at 37°C. Colony morphology differs slightly between media. On blood agar, both smooth, α-hemolytic and rough, nonhemolytic colonies may be found. It grows readily on most simple media. All species of *Listeria* will survive and grow when placed at 1°C to 5°C in a refrigerator, which will enable

the isolation of the agent from contaminated samples because of this kind of selectivity. However, time seldom allows this method to be used in a health-monitoring laboratory. Therefore, samples are usually placed in an enriched medium (e.g., Listeria Enrichment Broth, which is commercially available from various producers). In general, the selective principle is based on the addition of 0.2% thallium acetate or 25% to 75% tellurite salts and eventually 4 mg/l of nalidixic acid to a serum broth. The broth is incubated at 30°C for 48 h, preferably under microaerophilic conditions. The broth then is streaked on a selective-indicative agar or, alternatively, only an enriched agar, which are also commercially available. Alternatively, PALCAM (Phenol Red Acriflavin Lithium Chloride Columbia Agar with Mannitol) agar (see Table C.12) may be used.

From affected animals, the affected sites should be sampled. From healthy animals, the nose, trachea, cecum, and genitals should be sampled. A suitable procedure is to drop the piece of vagina that is cut off during sampling into an enrichment broth.

Different species of *Listeria* may be differentiated according to fermentation assays (Table 8.6). The kit API Listeria (bioMérieux) also identifies *Listeria* species within 24 h. Enzyme-linked immunosorbent assay (ELISA) has been used to verify the presence of *L. monocytogenes* in food samples, but because of cross-reactions with several other Gram-positive bacteria, this has thus far not been applied in laboratory animal bacteriology. PCR is widely used for identification.

8.10 Erysipelotrichidae

The pathogenic *Erysipelothrix* may be confused with the nonpathogenic *Lactobacillus*, from which it, however, can be differentiated by microscopy. *Erysipelothrix* grows as long, slender rods, and often filaments are formed; lactobacilli are straight and occasionally coccoid rods. A simpler differentiation can be based on *Erysipelothrix* producing H_2S, which lactobacilli do not produce. Tests for H_2S production must be performed in a triple-sugar iron slant (see Table C.9). In principle, only one species, *Erysipelothrix rhusiopathiae*, should be considered. It may be found in the wild rat population and may occasionally be the cause of arthritis in laboratory rats. Rats infected with *E. rhusiopathiae* show increased activity of β-lymphocytes.[48] Twenty-two serovars of *E. rhusiopathiae* have been described,[49] but probably only type 1 and type 2 are relevant. *Erysipelothrix rhusiopathiae* are slender or slightly curved long rods with a tendency to form long filaments. It is easily grown on simple, but enriched, media (e.g., chocolate or blood agar). After 24 h of microaerophilic, aerobic, or anaerobic cultivation at 37°C, pinpoint colonies are formed. Prolonged incubation does not increase the colony size. Selective enrichment is generally not performed, but brain heart infusion broth with 1% glucose is usable for propagation,

Table 8.6 Differentiation of *Listeria* spp.

	CAMP test		Acid from				
	S. aureus	***R. equi***	Mannitol	a-Methyl-D-mannoside	L-Rhamnose	Sucrose	D-Xylose
L. monocytogenes	+	–	–	+	+	–	–
L. innocua	–	–	–	+	d	d	–
L. seeligeri	+	–	–	d	–	?	–
L. welshimeri	–	–	–	+	d	?	+
L. ivanovii	–	+	–	–	–	d	+
L. grayi	–	–	+	+	–	–	–
L. murrayi	–	–	+	+	d	–	–

d = differs between strains.

Table 8.7 Biochemical Reactions for *Erysipelothrix rhusiopathiae*

Aerobic growth	+
Anaerobic growth	+
Motility	–
Catalase	–
Gas from glucose	–
Acid from	
Arabinose	–
Galactose	+
Lactose	+
Maltose	+
Mannitol	–
Melezitose	–
Melibiose	–
Raffinose	–
Salicin	–
Sorbitol	–
Trehalose	–
VP	–
NO_3 reduction	–
Aesculin hydrolysis	–
Arginin hydrolysis	+

VP = Voges–Proskauer test.

although other agents may propagate as well from mixed infections. In healthy animals, the nose seems to be the sample site of choice. From affected animals, the affected sites should be sampled. Confirmation of the diagnosis of *E. rhusiopathiae* may be made according to the reactions given in Table 8.7. Alternatively, the API Coryne kit (bioMérieux) may be applied, although it should be noted that identification solely based on this kit appears unreliable. PCR may be used for direct diagnosis on samples.[50]

8.11 Clostridiaceae

8.11.1 Impact on the host

Various genera of *Clostridium* are ubiquitous members of the microbiota, and only a few of them have a well-documented impact on the host. Some of them, such as *Clostridium perfringens* and *Clostridium difficile*, are cultivable on solid media; others, such as *C. piliforme*, are only cultivable by special means, and yet others, such as segmented filamentous bacteria (SFB) are yet to be cultivated and therefore are termed "Candidatus," in

this case, *Candidatus savagella.* The ASF 356 of the altered Schaedler flora is closely related to *Clostridium propionicum*; the ASF 356, ASF 492, and ASF 502 are all part of the *Clostridium* cluster XIV; and ASF 500 probably is related to *Clostridium* and at least is a Firmicutes species.[25]

8.11.1.1 Clostridium difficile *and* C. perfringens

Antibiotic-associated colitis may be induced in hamsters, guinea pigs, and occasionally in rabbits because of treatment with a range of antibiotics, (e.g., β-lactams, tetracyclines, clindamycin, gentamicin, lincomycin,[51] and sulbactam/cefoperazone[52]). This condition has previously most often been associated with *C. difficile*, but *C. perfringens* may seem equally important, eventually in connection with *E. coli*.[53–54] In guinea pigs, it is occasionally associated with *C. perfringens* type E, and in rabbits, especially after treatment with clindamycin, it may be caused by *Clostridium spiroforme*. In all cases, the condition is toxin mediated. The toxin A of *C. difficile* plays a more important role in the pathogenesis than toxin B. Both differ from the iota toxins of *C. perfringens* type E and *C. spiroforme*, which are similar to one another and may be neutralized with the same polyvalent antibodies. The symptoms occur within 10 days after treatment and include ruffled fur, dehydration, diarrhea, and death.

8.11.1.2 Clostridium spiroforme

Lethal enteritis in weanling rabbits without a previous history of antibiotic treatment may be caused by iota toxin-producing *C. spiroforme*. Actually, although fairly underestimated, this agent may be extremely important as the cause of rabbit enteritis, probably favored by maldigestion, other infectious agents, or nutritional factors. In one investigation, the bacterium was detected by Gram stain in 52.4% of 149 cecal samples and iota-like toxin in 7.4% from commercial rabbits showing clinical signs of enteritis complex; from 29 strains of *C. spiroforme* tested, 26 were toxigenic, originating from 24 of 29 rabbitries.[55] In 13.4% of the samples, *C. spiroforme* was present as the only known disease agent.[55] Intrinsic or acquired antimicrobial resistances are diffuse in the *C. spiroforme* population, and prevention is an option rather than treatment.[56]

8.11.1.3 Clostridium piliforme

The sole presence of the agent *C. piliforme* in the animal organism, whether causing disease or not, is termed *C. piliforme infection,* while Tyzzer's disease describes a condition in which pathological changes are present in the individual because of infection with *C. piliforme.* In mice, this highly lethal disease is characterized by multiple focal necroses of the liver, which macroscopically are observed as white spots. Inside the cells of such foci, long, thin, slender bacteria are found. These bacteria are also found in huge numbers in the alimentary tract, especially in the ileum and cecum. Tyzzer

proposed the name *Bacillus piliformis* for this agent,[57] and for many years, this name was commonly used, although never formally listed. Today, molecular techniques have allocated this agent to the genus *Clostridium* and it has been renamed *Clostridium piliforme*.[58] Tyzzer's disease has also been described in various other species, including rabbits,[59] Mongolian gerbils,[60,61] and Syrian and Chinese hamsters,[62,63] in which the symptoms are similar to those for mice. In mice, hamsters, and gerbils, it should be regarded as a fatal disease characterized by high mortality in the colony. Also, in rabbits the disease is often peracute with no symptoms prior to finding dead rabbits in the colony. In rats, however, Tyzzer's disease is a mild disease of weanlings connected with megaloileitis, multiple focal necroses of the liver, and single necroses in the myocardium.[64,65]

The zoonotic potential of this agent is discussed to some extent because it has been isolated from patient infected with human immunodeficiency virus (HIV).[66] Resistance to development of Tyzzer's disease may be because of genetic traits. Inbred strains of rats carrying MHC (major histocompatibility complex) haplotype RT1.A_l seem to be resistant; a high incidence may be found in rats carrying MHC haplotype RT1.A_u or RT1.A_k.[65,67] Also, some mouse strains, such as ICR, seem to be more susceptible than other strains of mice, which seems to be related to differences in levels of neutrophils, natural killer cells, macrophages,[68] and the inflammatory cytokines IL-6[69] and IL-12.[70] The organism probably persist in the gut epithelium of healthy animals. The prevalence, determined as the number of individuals with antibodies in infected rat and mouse colonies, varies, but it is often more than 50%.[71] In infected rabbit and gerbil colonies, the presence of antibodies is usually connected with clinical disease, while antibodies seem to disappear from the colony as mortality declines, but they may appear—depending on the assay—in the absence of both clinical symptoms and detectable organisms.[72] In Europe, antibodies against *C. piliforme* are detected frequently in rat colonies.[73]

A considerable range of different types of research interference may be expected, especially in animals with clinical Tyzzer's disease. This is mostly because of dysfunction of the liver and pathological changes found in the intestines, liver, and myocardium. However, interference may also be observed in nonaffected animals carrying this agent; for example, the half-life of trimethoprim may be prolonged in mice suffering from acute Tyzzer's disease and return to normal in the recovered mice, while the half-life of warfarin, which is also prolonged during acute Tyzzer's disease, never returns to the values observed in noninfected mice.[74] The agent may cross the placental barrier.[75]

8.11.1.4 Segmented filamentous bacteria (Candidatus savagella)

Segmented filamentous bacteria in mice colonize the ileum shortly prior to weaning,[76] in which they have a preference for the follicle-associated

species overlying the Peyer's patches[77]; in rabbits, they adhere more to the absorptive villi.[78] They have a complex life cycle as both endospores and vegetative bacteria.[79] As SFBs were first described in arthopods, these have precedence in terms of nomenclature, and the name *Candidatus arthromitus* should be reserved for SFBs in the Lachnospiraceae family, while *Clostridium* cluster I-related SFBs (i.e., those that may be found in rodents) should be referred to as *Candidatus savagella*. Mouse- and rat-associated species are closely related, but still different from one another.[80] Whether and to which extent they are found in humans is unclear, and if so, the human prevalence is probably low among humans more than 1 year old.[77] It is important to note that the same mouse strain, but from different vendors or breeding facilities, might differ in terms of SFB status, as experienced for C57BL/6, BALB/c (Taconic mice SFB positive; Jackson Laboratories mice SFB negative) and NOD mice (Taconic and Jackson mice negative; Harvard Medical School variable status, but some positive).[81,82] Knowing the SFB status of experimental rodent models is thus of great importance because it strongly influences immune system development and function, such as immunoglobulin (Ig) A production, T-cell response, and intestinal T_h17 cell induction.[81,83–85] For example, recolonization of germ-free mice with SFBs induces a full reconstitution of all CD4-positive T cells, including T_h1, T_h2, T_h17, and T_{reg},[77] as well as increases the expression of MHC class II.[84] The first indication that their presence in the gut made a difference to the host was a study showing their ability to reduce epithelial adherence of *Salmonella*,[86] which seems related to the induction of T_h17.[87] As T_h17 is also important in models of Crohn's disease, an impact of SFBs on IBD models should be expected, and adding SFBs to a reconstituting microbiota enhances intestinal inflammation observed in the SCID (severe combined immunodeficiency) adoptive transfer model.[88] SFBs also drive arthritis development in K/BxN mice[89] and seem to protect female NOD mice against type 1 diabetes development, while male NOD mice have low incidence regardless of SFB status.[82]

8.11.1.5 Faecalibacterium prausnitzii

Faecalibacterium prausnitzii is a clostridia-related bacterium.[90] In humans, it is among the most abundant cultivable members of the gut microbiota, constituting 2–5% of the total microbiota, where it is an important producer of butyrate and probably plays an important role in breakdown of complex carbohydrates[91,92]; it does not seem to be a common inhabitant of the murine gut.[15] It was until 1996 classified as belonging to the *Fusobacterium* genus, but 16S ribosomal RNA (rRNA) gene analysis revealed that it was more closely related to *Clostridium* cluster III and IV; following further analysis, *Fusobacterium prausnitzii* was in 2002 reclassified as *F. prausnitzii*.[90,93] It is in general positively associated with a healthy gastrointestinal environment in humans, in which it is linked

to a protective effect against Crohn's disease.[94] In mice, oral feeding of *F. prausnitzii* reduces the severity of colitis induced by 2,4,6-trinitrobenzene-sulfonic acid (TNBS).[94] This may also be the case in mouse models of colitis induced by both multidrug resistance gene-deficient (*mdr1a knockout*)[95] and dextran sodium sulfate (DSS).[96]

8.11.2 Characteristics of the agent

8.11.2.1 Clostridium difficile *and* C. perfringens

After 48 h of incubation on blood or chocolate agar, colonies of *C. difficile* are 2 to 4 mm in diameter, slightly raised, flat, and spreading and have a rhizoid edge. On cycloserine-cefoxitin egg yolk CCEY agar (see Table C.13), they are larger. Colony morphology among other clostridial types differs. *Clostridium difficile* mostly, but not always, stains clearly gram positive. In fact, some other clostridial species, *Clostridium clostridioforme* and *Clostridium sphenoides* mostly, stain Gram negative. Spores of *Clostridium* spp. may differ according to Figure 8.1. Colonies of *C. perfringens* show a typical double zone of hemolysis on blood agar, with the inner zone fully hemolyzed. The colonies are rather large and normally smooth but may also appear as rough. The cells are large with parallel sides, and spores are usually not observed in standard media. Chocolate agar modified to anaerobic cultivation (see Table C.6) is suitable for initial cultivation of more easily cultivable species, which, of course, must be performed according to the principles described for anaerobic cultivation. It is wise to suppress nonsporogenic bacteria in fecal and intestinal samples by incubating the sample with 50% ethanol for 1 h before inoculation on the anaerobic chocolate agar. For *C. difficile*, a selective agar such as the CCEY agar (see Table C.13) may be used. All cultivable clostridia may be grown at 37°C, but *C. perfringens* grows better at 43°C. In affected animals, sampling should be performed from the affected parts of the digestive system. In healthy animals, fecal pellets would be the most appropriate.

Some aerotolerant *Clostridium* spp. do exist; the most important one is *C. perfringens*, but as a rule of thumb, only species that are obligate anaerobic should be subjected to *Clostridium*-specific identification in the first place. The spores should be characterized according to Figure 8.1. A helpful tool is the inoculation of the isolates on egg yolk agar (see Table C.14).

Identification may be performed according to Table 8.8, which will not fully identify all species of *Clostridium*. However, it should be possible to differentiate *C. difficile* and *C. perfringens* clearly from other *Clostridium* spp. The commercial kit API 20A (bioMérieux) will also be helpful in the identification process. In gas chromatography on norleucine-tyrosine broth culture, *C. difficile* produces both caproic acid and *p*-cresol, which no other *Clostridium* does.[97] *Clostridium perfringens* may, in Table 8.8, be confused

Table 8.8 Differentiation of *Clostridium* spp.

	Spore type[a]	Egg yolk agar		Gelatin hydrolysis	Indole	Acid from					
		Lecithinase	Lipase			Glucose	Maltose	Lactose	Sucrose	Salicin	Mannitol
C. difficile	O S	–	–	d[b]	–	+	–	–	–	d	+
C. butyricum	O S	–	–	–	–	+	+	+	+	–	–
C. chauvoei	O S	–	–	d	–	+	+	+	+	–	–
C. clostridioforme	O S	–	–	–	d	+	+	d	+	d	–
C. septicum	O S	–	–	+	–	+	+	+	–	+	–
C. histolyticum	O S	–	–	+	–	–	–	–	–	–	–
C. subterminale	O S	–	–	+	–	–	–	–	–	–	–
C. perfringens	O S	+	–	+	–	+	+	+	+	–	–
C. bifermentans	O S	+	–	+	+	+	+	–	–	d	–
C. limosum	O S	+	–	+	–	+	–	–	–	–	+
C. novyi	O S	+	d	+	–	+	d	–	–	–	–
C. sordellii	O S	d	–	+	+	+	+	–	–	–	–

C. sporogenes	O S	–	+	+	–	+	+	–	–	d	–
C. botulinum	O S	–	+	+	–	+	d	–	–	d	–
C. cadaveris	O T	–	–	d	+	+	–	–	–	+	–
C. innocuum	O T	–	–	–	–	+	–	–	d	+	–
C. paraputrificum	O T	–	–	–	–	+	+	+	+	+	–
C. tertium	O T	–	–	–	–	+	+	+	d	+	+
C. ramosum	R/O T	–	–	–	–	+	+	+	+	+	d
C. sphenoides	R S/T	–	–	d	d	+	+	d	d	+	+
C. tetanii	R T	–	–	+	d	–	–	–	–	–	–

[a] O = Oval, R = Round, T = Terminal, S = Subterminal.
[b] d = differs between strains

with some closely related species (e.g., *C. clostridioforme, C. butyricum,* and *C. septicum*). *Clostridium perfringens* is, however, nonmotile, but most other *Clostridium* spp. are motile. Furthermore, screening for lecithinase activity on egg yolk agar (see Table C.14) should clearly differentiate *C. perfringens* from other types of clostridia. Identification by antibodies is possible using latex agglutination for *C. difficile* or by the Immunocard® (Meridian Diagnostics, Cincinnati, OH, USA) (i.e., a quick enzymatic antibody test). *Clostridium difficile* may be identified by nucleic acid amplification tests based on the detection of toxin genes, and these tests are commercially available.[98]

8.11.2.2 Clostridium spiroforme

Clostridium spiroforme may join to form tight coils or spiral configurations (see Figure 3.2, item 11). This is the main characteristic used for identification of this agent, and *Clostridium* spp. having this configuration may be diagnosed right away as *C. spiroforme.* This, however, is not sufficient for the diagnosis of *C. spiroforme*-mediated diarrhea in rabbits because only iota toxin-producing *C. spiriforme* may be regarded as pathogenic. Furthermore, it should be noted that *C. spiroforme* does not appear as such in fecal specimens, but rather as semicircular bacteria.[99] *Clostridium spiroforme* may be cultivated on *Perfringens* Agar Base (Oxoid, UK) supplemented with Shahidi-Ferguson-*Perfringens* Selective Supplement (Oxoid) and incubated in anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) at 37°C for 24–48 h.[56] A presumptive diagnosis of *C. spiroforme*-mediated diarrhea in rabbits may be made by directly Gram staining feces from affected rabbits to look for semicircular bacteria. Feces with such bacteria should be further cultivated according to the principles described previously. *Clostridium spiroforme* is easily identified by PCR.[100]

8.11.2.3 Clostridium piliforme

Clostridium piliforme is a thin rod approximately 0.3 to 0.5 µm wide and 8 to 10 µm long. In spore-forming cells, the spores may be seen as thickenings in the end, which give the agent the shape of a drumstick. The spores are elliptical and are often referred to as "rocket spores." They may be stained by spore staining, but the simplest way to find them is to look for them in an immunofluorescence-stained smear, in which they are easily observed. In histological slides, bacterial cells are grouped as bundles (see Figure 3.2, item 5). Smears may be prepared and stained by either immunofluorescence or peroxidase-antiperoxidase (PAP) techniques as described in Chapter 4. Samples from the ileum are made by cutting an approximately 1-cm piece of the ileum, inverting it, and washing the inner surface with distilled water. Then, smears are made as described in Chapter 4. A simple stain may be prepared from Giemsa solution. The slide is fixed for 20 s in absolute methanol and then stained for 2 min in

undiluted Giemsa's azur eosin methylene blue solution (Merck Millipore, Germany). Using a high titer, antibody immunofluorescence staining is superior to both PAP and Giemsa staining.

Attempts to cultivate *C. piliforme* by ordinary bacteriological methods have thus far been unsuccessful[101]; therefore, cell cultures, such as the mouse-embryo fibroblast cell line 3T3, the mouse–connective-tissue-originated cells L-929 or the mouse liver cells National Collection of Type Cultures (NCTC) 1469. An initial decrease in the number of bacteria is noted after inoculation of the cell layer with 2.6×10^5 organisms followed by a peak bacterial count at 48 h.[102] As an alternative, the suspension may be inoculated aseptically into embryonated eggs. Eggs 6 to 9 days old are inoculated in the yolk sac with infected material. The viability is determined by candling three times per day. Embryonic death within 24 h is disregarded. Yolk sac from embryos dying later can be harvested and frozen at –80°C. A 20% phosphate-buffered saline (PBS) suspension should be examined for the presence of the agent.[103] The ileum, liver, or myocardium should be searched for the presence of *C. piliforme*. *Clostridium piliforme* is easily identified in even low numbers in feces and gut content by PCR.[104–106]

Both immunofluorescence assay and ELISA are usable for screening for antibodies against *C. piliforme*. Care should be taken not to use too low a cutoff value, and testing should be performed primarily on animals more than 10 weeks old. High titers may develop as early as 10 days postinfection and reach a maximum within 25 days. It should be noted that the specificity of serology for *C. piliforme* can be rather low, and it can be difficult to confirm a seropositive diagnosis by PCR.[72]

*8.11.2.4 Segmented filamentous bacteria (*Candidatus savagella*)*

All attempts to cultivate SFBs have been unsuccessful, but they can be directly detected by microscopy of gut epithelium as segmented filamentous bacteria 0.7 to 1.8 µm wide and up to 80 µm long[77] (see Figure 3.2, item 12) attached to the epithelium through characteristic holdfast structures. However, by treating the intestinal content of mouse carriers with ethanol and chloroform and subsequently inoculating the homogenates into germ-free mice, SFB monoassociated mice can be produced.[107] Snel et al.[108] showed by 16S rRNA gene comparison that SFBs are phylogenetically closest related to, but clearly distinct from, *Clostridium* cluster I and proposed the provisional genus name *Candidatus arthromitus*. Later, Thompson et al.[109] found that SFBs of arthropod origin are more closely related to family Lachnospiraceae, thus being phylogenetically distant to SFBs of vertebrate origin. SFBs of vertebrate origin (*Candidatus savagella*) can be quantified relatively easily using specific quantitative PCR (SYBR® assay, primers SFB736 forward: GACGCTGAGGCATGAGAGCAT and

SFB844 reverse: GACGGCACGGATTGTTATTCA).[82] An SFB standard can be produced by cloning and PCR.

8.12 Lachnospiraceae

Lachnospiraceae are Gram-positive, non-spore-forming, obligately anaerobic, motile, rod-shaped, pectin-fermenting bacteria harbored in the gut of various species, including rodents, and they seem to be quantitatively and positively correlated to improved glucose tolerance in leptin-deficient obese mice.[110] Genera found in mice are *Coprococcus, Dorea, Rosebouria*, and *Robinsoniella*, for example.[15] They are mostly observed in high-throughput sequencing-base data sets.

8.13 Eubacteriaceae

Eubacterium spp. may stain either as Gram-positive or -negative. They belong to the family Eubacteriaceae and may be either motile or non-motile. They do not seem to be dominant in the gut microbiota of mice,[15] but they are found in rats, and in diabetes-prone biobreeding (BB) rats, they are more abundant.[111] They may be cultivated by incubation in a BACTEC™ anaerobic bottle and a BACTEC automated instrument (Becton Dickinson, Franklin Lakes, NJ, USA) for 10 days, and they may grow on blood agar plates at 37°C in an anaerobic atmosphere.[112] The biochemical characteristics of the organism may be determined with the API 20A (BioMerieux).[112] The ASF 492 as part of the altered Schaedler flora has been shown by sequencing to be *Eubacterium pexicaudatum*.[25]

8.14 Ruminococcaceae

Ruminococcus spp. are anaerobic, Gram-positive cocci that are abundant in both rats[111] and mice.[15] In diabetes-prone BB rats, they are more abundant.[111] ASF 502 of the altered Schaedler flora is closely related to *Ruminococcus gnavus*.[25]

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chapter nine

Bacteroidetes

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9.1 Introduction

Bacteroidetes are anaerobic rods of the gut and skin that do not form endospores. They account for 25–60% of the bacteria in the gut[1,2]; therefore, together with Firmicutes, they cover the vast majority of gut inhabitants. As knowledge of the abundance of such anaerobic phyla is limited, because high-throughput sequencing techniques have only been available for a relatively short time, we probably have much to learn, but it is likely that the size of the Bacteroidetes fraction can be of importance for a range of animal models. For example, the balance between Firmicutes and Bacteroidetes is important for the development of obesity and glucose intolerance in leptin-deficient obese mice, which harbor significantly more Firmicutes and fewer Bacteriodetes compared to their wild-type and heterozygous littermates.[3] Further, mice that by knockout are made unable to produce the active form of vitamin D or the vitamin D receptor have more bacteria from the Bacteroidetes and Proteobacteria phyla and

fewer bacteria from the Firmicutes and Deferribacteres phyla in their feces compared with wild-type mice.[4] Identification of Bacteroidetes members are generally done by polymerase chain reaction (PCR) (e.g., quantitative PCR [qPCR]) or sequencing-based techniques, though several members can be relatively easily cultivated by classical, culture-based microbiological techniques.

The order Bacteroidales contains members with a well-described impact on rodents. In addition, rodents harbor Porphyromonadaceae, such as *Barnella* spp., *Odoribacter* spp., and *Parabacteroides* spp.[5] Also, cilia-associated respiratory (CAR) bacillus is a Bacteroidetes species at present systematized within Sphingobacteriales.

9.2 Bacteroidales

9.2.1 Bacteroidaceae

9.2.1.1 Impact on the host

Bacteroides spp., as in all other mammals, form an important part of the Bacteroidetes fraction of the rodent gut.[5] These Gram-negative, anaerobic, but transiently aerotolerant bacteria are important for the processing of complex molecules to simpler ones in the gut,[6] and complex glycans are their key source of energy.[7] A diet dominated by proteins increases the *Bacteroides* numbers; a diet rich in carbohydrates increases *Prevotella* numbers.[8] Growth of enterobacteria, enterococci, and *Bacteroides fragilis* in mice is reduced by feeding *Bifidobacterium* spp.[9] In the biobreeding (BB) rat, a spontaneous type 1 diabetes model, the fecal microbiota differs between diabetic and nondiabetic animals[10] and contains an increased number of *Bacteroides* spp. prior to diabetes onset.[11] In an azoxymethane/dextran sodium sulfate (DSS) model of colorectal cancer, tumor-bearing mice showed enrichment in operational taxonomic units (OTUs) affiliated with members of the *Bacteroides*.[12]

Bacteroides fragilis is normally considered a gut commensal, but there seems to be a clear differentiation on the influence of the host between whether the strains are toxin producing or not. In humans, it is known to be able to cause septicemia or wound infections, which can be problematic because *B. fragilis* is also known to be relatively antibiotic resistant (e.g., because of the production of β-lactamase).[13] This is probably not as relevant for rodents because ampicillin in mice, generally naïve to antibiotic treatment, results in a gut with low abundance of any form of bacteria.[14] It has also been incriminated as involved in diarrhea and inflammatory bowel disease (IBD) in humans, and feeding gnotobiotic mice *B. fragilis* toxins does cause symptoms of this.[15] Also, enterotoxigenic strains of *B. fragilis* cause lethal colitis in Mongolian gerbils,[16] and its toxins strongly induce colonic tumors in multiple intestinal neoplasia (Min) mice.[17] On the other

hand, *B. fragilis* polysaccharide A (PSA) protects against colitis induced by *Helicobacter hepaticus* in mice, probably because of the prevention of interleukin (IL) 17 secretion.[18] PSA is a strong stimulator of the dendritic cells in mice; therefore, *B. fragilis* PSA is an important part of gut immune system maturation and keeping a proper Th1/Th2 balance, as well as for the inflammatory gut response to pathogens.[19] Feeding the maternal immune activation (MIA) mouse model of autism *B. fragilis* reduces symptoms of autism, which is probably linked to the normalization of a specific gut metabolite.[20] *Bacteroides vulgatus* seems to enhance IBD in HLA-B27 transgenic rats[21] and IL-10 knockout mice.[22] ASF 519, which is contained in the altered Schaedler flora, is related to, but still different from, *B. distasonis*.[23] *Bacteroides uniformis* ameliorates some of the metabolic and immunological dysfunctions induced by a high-fat diet in diet-induced obese mice.[24]

9.2.1.2 Characteristics of the agent

Bacteroides spp. can be cultivated anaerobically at 35–37°C for 3 days on 5% blood agar (see Table C.1). *Bacteroides fragilis* is resistant to kanamycin (1000 µg/disk), vancomycin (5 µg/disk), and colistin (10 µg/disk), and it will grow in 20% bile, in which many other *Bacteroides* spp. do not grow. The kits RAPID II Ana (Oxoid, UK), API 20 A (bioMérieux, France), and API-ZYM (bioMérieux) may be used for identification with a specificity of 60–80%.[25] Previously, gas liquid chromatography has been used for identification, but today, the strongest tool seems to be various types of PCR and DNA sequencing.[26]

9.2.2 S24-7

S24-7 is a hitherto uncultivated family within Bacteroidales for which a high abundance in mice in one study correlated to development of type 2 diabetes after high-fat feeding of C57BL/6 mice[27]; in another study with BALB/c mice, it seemed to decrease after high-fat feeding.[28] Abundance of S24-7 seems to correlate to late- or no onset of type 1 diabetes as well as a high number of regulatory T cells in nonobese diabetic mice.[29] It is also correlated with better memory performance in BALB/c mice.[28]

9.2.3 Prevotellaceae

9.2.3.1 Impact on the host

Prevotella spp. are Gram negative, nonmotile, and rod-shaped to coccoid and prefer anaerobic conditions, although they are also found in the oral cavity. In an azoxymethane/DSS model of colorectal cancer, tumor-bearing mice showed decreases in abundance of species affiliated with members of the Prevotellaceae[12]; in contrast, abundance of Prevotellaceae, perhaps restricted to one unclassified genus, in the gut of leptin-deficient

obese mice correlated to impaired glucose tolerance.[30] *Prevotella copri*, which has been correlated with the development of arthritis in humans, may increase the severity of DSS-induced colitis in mice.[31] *Caspase-3* knockout mice exhibit a lower inflammatory response to DSS induction of colitis compared to wild-type mice, but this protective effect of the mutation decreases by cohousing knockout mice with wild-type mice, which significantly increases the abundance of *Prevotella* spp. in the knockout mice.[32]

9.2.3.2 Characteristics of the agent

Prevotella spp. can be cultivated anaerobically at 37°C for 2 days and be maintained on Eggerth Gagnon (EG) agar (Merck, Germany) supplemented with 5% horse blood at 37°C in an atmosphere containing 100% CO_2. The kits API 20 A, API Zym, and API An Ident (bioMérieux) may be used for differentiation and identification.[33] Various types of PCR and sequencing target genes may be used for identification.[26]

9.2.4 Rikenellaceae

Alistipes spp. of Rikenellaceae can be cultivated and identified as described for *Bacteroides* spp., and they resemble members of the *B. fragilis* group as they are resistant to bile and exhibit resistance to vancomycin, kanamycin, and colistin, but they produce a brown pigment on media containing hemolyzed blood.[34] They have a strong impact on the fecal metabolic profiles in mice[35]; for example, in a mouse model of autism induced with in utero exposure to valproic acid, a high level of *Alistipes* in the gut correlated to a low level of serotonin in the ileum.[36] Grid floor housing increases the abundance of *Alistipes* spp. in mice.[37]

Mice harbor other Rikenellaceae members.[5] Some of these may be *Rikenella* spp., which in the previously mentioned mouse model of autism induced with in utero exposure to valproic acid correlated negatively to the cecal levels of butyric acid.[36]

9.2.5 Porphyromonadaceae

Rodent guts may harbor Porphyromonadaceae such as *Barnella* spp., *Odoribacter* spp., and *Parabacteroides* spp.,[5] but their impact is not clear.

9.3 Flavobacteriales

The genera *Flavobacterium*, *Chryseobacterium*, and *Cloacibacterium* may be shown in some, but not all, mice by sequencing.[5] *Flavobacterium* spp. are occasionally isolated from both laboratory rodents and rabbits. Although

they may be part of a mixed flora in wounds, there is no indication that they should be of any importance as pathogens or have any severe impact on research. The cells are pleomorphic and may show filamentous forms. Colonies are 1 to 2 mm and often pigmented.

9.4 Sphingobacteriales

Infection with CAR bacillus has been reported in mice, rats, and rabbits, and in farm animals such as pigs and goats, in Europe, the United States, and Japan. Determination of gene sequences have shown that although the rabbit isolates of CAR bacillus show a greater similarity with *Helicobacter*,[38] isolates from rats and from mice have been related to *Flavobacterium*,[39,40] although they also show some similarity with *Flexibacterium*, which is also a genus within Bacteroidetes.[39] As its closest relatives are *Flexibacterium sancti* and *Flavobacterium ferrugineum*,[39] the latter of which today has been reclassified as *Terrimonas ferruginea*,[41] and both of these are Sphingobacteriales, it is probably most correct to regard it as a species within this order. CAR bacillus isolates from rats and mice; and rabbits, on the other hand, are host specific and should be regarded as being different bacteria that belong to distinct genera.

It is known from sequencing that within Sphingobacteriales mice also harbor *Sediminibacterium* spp. and *Aquiflexum* spp.,[5] but their influence on rodent animal models remains unknown.

9.4.1 Cilia-associated respiratory bacillus

9.4.1.1 Impact on the host

The pathogenicity of CAR bacillus in rodents is not fully clarified, and infected rodents are usually asymptomatic,[42] but the agent is regarded as the cause of *chronic respiratory disease* in rats; that is, this disease is a highly contagious epizootic, slowly progressive, and uncontrollable disease in which symptomatic rats histopathologically show various degrees of pulmonary changes, such as mucopurulent exudate and severe peribronchial lymphoid cuffing.[43] Clinical signs, if present, may include weight loss, rough hair coat, wheezing, and rales. The same clinical picture may be observed in mice, with BALB/c mice being susceptible and C57BL/6 resistant,[44] which seems related to differences in IL-10 and interferon gamma (IFN-γ) responses.[45] There are no reported differences in the susceptibility between rat strains, but different strains of the agent are more or less virulent. Severity of symptoms during experimental infection seems to be worse if an isolate of the same rodent species is used.[42,46] The colony prevalence for laboratory rat colonies is less than 1%,[47] or even 0% in Europe[47] and Japan,[48] but in pet rats, it is close to 100%.[49]

9.4.1.2 Characteristics of the agent

Cultivation in traditional bacteriological media is difficult. For several years, propagation by inoculation of embryonated chicken eggs via the allantoic route has been used. The agent may also be propagated in Dulbecco's or Eagle's minimum essential medium supplemented with 10% fetal calf serum. In addition, 20% hamster tracheal organ culture soup may be added. Isolates from rats are approximately 0.2 µm wide and 4 to 8 µm long with a triple-layer cell wall and bulbous ends. Cells are Gram negative, not acid fast, not spore forming, and heat labile (56°C for 30 min). CAR bacillus is motile but lacks structures resembling flagella, pili, or axial filaments. The rabbit bacilli are smaller and form fewer aggregates during propagation.[50]

In paraffin-embedded infected lungs, the filamentous bacteria may be detected by various silver stains on the border of the tracheal and bronchial epithelium (e.g., Warthin-Starry) or more specifically by using either indirect immunofluorescence or an immunoperoxidase staining technique based on the labeled streptavidin biotin method and 3-amino-9-ethylcarbazole (AEC) as substrate[51] (see Chapter 4). Antibodies to CAR bacillus may be detected in the early stage of the infection by either immunofluorescence assay (IFA)[52] or an enzyme-linked immunosorbent assay (ELISA).[52] The antigen may be prepared from allantoic fluids containing approximately $2 \cdot 10^8$ per milliliter of the agent washed with sterile phosphate-buffered saline (PBS), resuspended 1:5 in sterile coating buffer, and sonicated. Dilutions (1:40 or 1:80) are used as antigen solutions and coated on the surface of microtiter plates as described in Chapter 5.[52] CAR bacillus may be detected by PCR on infected lung tissue or on swabs from the nasal or oral cavity on day 3 postexposure.[53–56]

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chapter ten

Proteobacteria

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10.1 Gammaproteobacteria

10.1.1 Enterobacteriaceae

Enterobacteriaceae are catalase positive and oxidase negative, and the carbohydrate utilization is fermentative. Both motile and nonmotile species occur.

10.1.1.1 Impact on the host

Some Enterobacteriaceae (e.g., *Proteus*, *Morganella*, and to some extent *Escherichia*) are commensal members of the gut microbiota; others (e.g., *Salmonella* and *Yersinia*) are highly undesirable facultative pathogens in laboratory animal colonies. They are the primary carriers of the Toll-like receptor 4 stimulating lipopolysaccharides (LPSs) in their cell walls (Chapter 6), and when reducing type 1 diabetes by preweaning treatment of nonobese diabetic (NOD) mice with vancomycin, one of the observations in the puppies is an increase in the abundance of Proteobacteria.[1]

10.1.1.1.1 Escherichia Most rodents, with the important exceptions of hamsters and guinea pigs, carry *Escherichia coli* as a normal inhabitant in the cecum and the large intestines, but it has been shown to enhance irritable bowel disease (IBD) in HLA-B27-overexpressing rats,[2] although it has also been shown that *E. coli* Nissle stabilizes the enteric barrier in mice.[3] In rabbits, in which it is not a normal inhabitant but an important pathogen, it may cause disease in stressed animals, and the same may be the case in species in which it is a normal inhabitant. Enteritis is the most common of such diseases caused by *E. coli.* In rabbits, the strains of *E. coli* are not toxin producing,[4] rendering vaccination with commercially available pig vaccines of little use in a rabbit colony. It may also be involved in other types of disease (e.g., cystitis, pyelonephritis, metritis, pneumonia, and local inflammation). In rabbit colonies, outbreaks of colibacillosis may be connected with high losses during a period, the main symptom being diarrhea. After a period, most outbreaks seem to stop by themselves. Often, *E. coli* is only secondary, and other infectious agents, such as rotavirus, are the main pathogen. In guinea pigs, fatal enteropathies may be caused by *E. coli* after fecal-oral transmission in poorly housed young animals. In lactating females, mastitis is occasionally caused by *E. coli.* In rat colonies, *E. coli* can be isolated from approximately 70% of cecal samples; intestinal samples from moribund rabbits lead to isolation in nearly 100% of the cases if *E. coli* is the cause. Other *Escherichia* spp. are not common inhabitants of rodents and rabbits.

10.1.1.1.2 Citrobacter *Citrobacter* spp. can be isolated from the intestines and genitals of a range of animal species. Main species isolated from rodents are *C. freundii* and *C. diversus*. In particular, *C. freundii* is a common finding in hamsters and guinea pigs; *C. diversus* is most common in rats. In most cases, these infections do not cause any symptoms, and the observed prevalences are rather low. In extreme cases, *C. freundii* may cause epizootic pneumonia and enteritis with high mortality in guinea pig colonies.[5] A third species, *C. rodentium*, formerly known as either *C. freundii* type 4280[6] (because of its identification number in the commercial kit API 10E), *C. freundii* type ANL[7] or *C. freundii* type Ediger,[8] has been of

major importance as an intestinal pathogen in male mice. Symptoms are most common in sucklings and weanlings, in which colonic hyperplasia and rectal prolapses, diarrhea, and dehydration are observed.[6–9] Some strains, such as CD-1 and C57BL/6, develop only subclinical symptoms; other strains, such as FVB/N and C3H/HeJ mice, are highly susceptible.[10] Various stressors influence both morbidity and mortality,[11] but generally high losses are not common during spontaneous infections. The composition of the gut microbiota has an essential impact on disease susceptibility[12]; for example, infected mice lacking segmented filamentous bacteria (see Chapter 8) exhibit symptoms of increased severity.[13] Prevalence rates are normally rather low; for instance, often the agent is detectable in less than 5% of the individuals from an infected colony.

10.1.1.1.3 Salmonella *Salmonella* spp. infect all species of warm-blooded animals. Until the introduction of barrier-protected breeding systems in the 1960s, *Salmonella* commonly ruined research projects involving the use of rodents, especially if the project involved the use of mice. In mice and rats, in which *Salmonella enterica* serotype Typhimurium is the most common, clinical disease is characterized by various grades of diarrhea.[14] During the first period of an outbreak, a number of sudden deaths caused by septicemia occur, but afterward, the conditions turn chronic, and a number of animals will act as persistent carriers. In the highly susceptible guinea pig, *Salmonella* Enteritidis is the most common species of serotype *Salmonella enterica*. Symptoms are similar to those in rats and mice; furthermore, in breeding colonies, abortions may occur.[15] Rabbits may suffer from salmonellosis, but this is rather uncommon. In colonies infected with *S.* Typhimurium or *S.* Enteritidis the prevalences observed mostly range above 50%, but several other species of *Salmonella* may be introduced through contaminated diets, staff, and so on; in such cases, the prevalences are often extremely low. For instance, *Salmonella* may be isolated from a single individual, but the diagnosis is impossible to confirm by additional isolations. Uterine infections—probably without passage of the placenta barrier—have been described for *Salmonella*.[16]

10.1.1.1.4 Other species *Yersinia pseudotuberculosis* and *Y. pestis* may in theory be isolated from rodents, but this is hardly to be regarded of any relevance in modern laboratory animal facilities. It should be noted that pseudotuberculosis in rats and mice is not caused by *Yersinia* but by *Corynebacterium kutscheri* (see Chapter 12). Four species of *Klebsiella*—*K. orthinolytica, K. oxytoca, K. planticola,* and *K. pneumoniae*—may be isolated from both rodents and rabbits. Generally, prevalences are rather low (i.e., 20% or less), and clinical affection is seldom observed, although in mice and guinea pigs, pneumonia and deaths caused by septicemia are observed in rare cases. High prevalences may be because of uncritical antibiotic

treatments in the colony.[17] Four species of *Enterobacter*—*E. aerogenes, E. agglomerans, E. cloacae, E. gergoviae,* and the closely related *Cronobacter sakazakii*—are known to be isolated from laboratory rodents. The most common is *E. cloacae,* which may show prevalences of 25% to 30%. Only one species of *Proteus*, *P. mirabilis*, is isolated from rodents. Prevalences vary from low to close to 100%, but it is not associated with disease. *Morganelli morganii*, *Edwardsiella* spp., *Hafnia* spp., *Klyuvera* spp., *Providencia* spp., and *Serratia* spp. are occasionally isolated from a low number of individuals in rodent colonies, but no disease symptoms are known to occur from this kind of infection.

10.1.1.2 Characteristics of the agent

Morphological descriptions of important members of Enterobacteriaceae are given in Table 10.1. Enterobacteriaceae are easily cultivated on either blood agar or MacConkey agar[8] (see Table C.15). If blood agar is used, a detergent for prevention of *Proteus* swarming should be included in the recipe (see Chapter 3). Enterobacteriaceae from laboratory animals easily grow after 24 h of aerobic incubation at 37°C, but isolation is also possible after microaerophilic or anaerobic incubation if this is necessary

Table 10.1 Colony Morphology of Important Enterobacteriaceae in Rodents and Rabbits after Incubation on Blood Agar

Species	Colony morphology (24 h)	Bacterial morphology
Citrobacter spp.	2- to 3-mm large, round colonies with a smooth edge and surface	Short (0.6- to 1.5-µm), slightly pleomorphic rods
Enterobacter cloacae	Large 2- to 4-mm mucoid colonies (less mucoid than *Klebsiella*)	Long, straight rods, 0.6–1.0 µm wide and 1.2–3.0 µm long
Escherichia coli	2- to 3-mm large, round colonies with a smooth edge and surface	Coliform rods (see Figure 3.2, item 7), but cocoid forms may occur
Proteus mirabilis	Swarms in waves to cover the whole agar	Coliform rods (see Figure 3.2, item 7)
Klebsiella pneumoniae	Large 3- to 4-mm mucoid, irregular colonies	Plump rods with a thick capsule; some individual cocci may be observed
Morganella morganii	2- to 3-mm flat colonies	Coliform rods
Salmonella spp.	2- to 3-mm flat, circular, gray colonies	Long (2- to 3-µm) rods
Yersinia pseudotuberculosis	0.5- to 2-mm smooth colonies with no clear edges (30°C)	Pleomorphic rods with rounded ends; staining is irregular

because of examinations for other bacteria. If the sole aim of the examination is to isolate *Yersinia*, cultivation at 25°C is recommended; 42°C is recommended if *Salmonella* is the main target. Samples for *Salmonella* should be propagated in a propagation broth if healthy animals are examined in routine investigations. Several such media are commercially available or can be prepared on site in the laboratory (e.g., selenite broth[9]) (see Table C.16). After a short incubation time, an indicative or selective agar, such as brilliant-green phenol-red lactose-sucrose (BPLS) agar (see Table C.17), is streaked from the broth. As propagated, *Salmonella* bacteria do not survive for too long in a broth; the incubation time of the propagation broth should be kept less than 24 h. Main sample sites for Enterobacteriaceae are the cecum, feces, and genitals, although they may be isolated from several other sites as well.

After growing pure cultures of all isolates, each isolate should be subjected to microscopy, tests for Gram properties, oxidase, catalase, motility, and ability to grow in KCN. If the isolate is a member of Enterobacteriaceae, identification may be performed according to Tables 10.2 to 10.5. However, identification of Enterobacteriaceae is generally much easier using a commercial test kit rather than in-house tests. Several commercial kits for Enterobacteriaceae are available, such as API 20E (bioMérieux, France), Enterotube II (Becton Dickinson, France), or Micro-ID (Thermo Scientific, Waltham, MA, USA). It should be kept in mind that these kits were not developed for laboratory animal use; therefore, the identification procedure will not reveal all rodent-specific bacteria. The API 20E identification codes for *C. rodentium* are 1604513, 1404513, 1204513, 1104513, and 1004513.

If only specific types of Enterobacteriaceae are searched for, a screening test should be included to limit the number of isolates that must be fully identified. Tests for motility and KCN resistance as proposed by Tables 10.2 to 10.5 are valuable tools and may be combined with cultivation on a lactose-sucrose agar in routine examination. For example, in the Federation of European Laboratory Animal Science Associations (FELASA) guidelines for health monitoring of laboratory animals,[18] only *C. rodentium* and *Salmonella* are mandatory agents, and both are lactose-sucrose negative; this seldom occurs for *E. coli*, which is the most common species of Enterobacteriaceae found in rats and mice. Detection of antibodies in serum samples is not a common tool for detection of Enterobacteriaceae in rodent colonies. Full identification of *Salmonella* includes determination of O and H antigens, which can be done by slide agglutination using polyvalent sera. In most countries, reference centers able to group *Salmonella* serologically are run on a national basis. *Salmonella* may be detected and identified by polymerase chain reaction (PCR) (e.g., as a multiplex solution).[19]

The US Centers for Disease Control and Prevention (CDC) recommend biosafety level 2 practices, containment equipment, and facilities for activities with cultures of, or clinical materials potentially infected

Table 10.2 KCN-Resistant, Motile Enterobacteriaceae. Found in Rodents and Rabbits

	Citrobacter		*Enterobacter*									*Serratia*	
	freundii	*rodentium*	*aerogenes*	*agglomerans*	*cloacae*	*gergoviae*	*sakazakii*	*Hafnia alvei*	*Kluyvera*	*Morganella morganii*	*Proteus mirabilis*	*liquefaciens*	*marquescens*
KCN (growth)	+	+	+	d	+	d	+	+	+	+	+	+	+
Motility (37%)	+	+	+	d	+	+	+	d	+	+	+	+	+
β-Galactosidase	d	+	+	+	+	+	+	d	+	–	–	+	+
Arginine decarboxylase	d	–	–	–	d	–	+	–	–	–	–	–	–
Lysine decarboxylase	–	–	+	–	–	d	–	+	d	–	–	d	+
Ornithine decarboxylase	–	+	+	–	d	+	d	+	+	+	+	+	+
Citrate	d	d	d	d	d	d	+	d	d	–	d	d	+
H_2S	d	–	–	–	–	–	–	–	–	–	d	–	–
Urease	d	–	–	d	–	+	–	–	–	+	+	–	d
Tryptophane deaminase	–	–	–	–	–	–	–	–	–	+	+	–	–
Indole	d	–	–	d	–	–	d	–	d	+	–	–	–
VP	–	–	d	d	d	+	d	d	–	–	–	d	d
Gelatin liquefaction	–	–	–	d	–	–	d	–	–	–	d	d	d
Glucose (gas)	d	+	+	+	+	+	+	d	+	+	+	d	d
Lactose	d	–	+	d	+	d	+	+	+	–	–	–	–
Sucrose	d	–	+	d	+	+	+	d	–	–	–	+	+
Mannitol	+	+	+	+	+	+	+	+	+	–	–	+	+
Inositol	–	–	+	d	d	d	d	–	–	–	–	d	d
Sorbitol	+	+	+	d	d	–	–	–	–	–	–	+	+
Rhamnose	+	+	+	d	d	+	+	d	+	–	–	–	–
Sucrose	d	–	+	d	+	+	+	–	–	–	–	+	+
Melibiose	d	–	+	d	d	+	+	–	+	–	–	d	d
Amygdalin	d	+	+	+	+	+	+	d	+	–	–	+	+
Arabinose	+	+	+	+	+	+	+	d	+	–	–	d	d

d = differs between strains; VP = Voges–Proskauer test.

Table 10.3 KCN-Sensitive, Motile Enterobacteriaceae. Found in Rodents and Rabbits

	Citrobacter diversus	*Edwardsiella tarda*	*Enterobacter*		*Escherichia coli*[a]	*Salmonella*
			agglomerans	*gergoviae*		
KCN (growth)	–	–	d	d	–	–
Motility (37%)	+	+	d	+	+	+
β-Galactosidase	+	–	+	+	+	–
Arginine decarboxylase	d	–	–	–	–	d
Lysine decarboxylase	–	+	–	d	+	+
Ornithine decarboxylase	+	+	–	+	d	+
Citrate	+	–	d	d	–	d
H_2S	–	+	–	–	–	d
Urease	d	–	d	+	–	–
Tryptophane deaminase	–	–	–	–	–	–
Indole	+	+	d	–	+	–
VP	–	–	d	+	–	–

continued

Table 10.3 (continued) KCN-Sensitive, Motile Enterobacteriaceae. Found in Rodents and Rabbits

	Citrobacter diversus	*Edwardsiella tarda*	*Enterobacter*		*Escherichia coli*[a]	*Salmonella*
			agglomerans	*gergoviae*		
Gelatin liquefaction	–	–	d	–	–	–
Glucose (gas)	+	+	+	+	+	+
Lactose	d	–	d	d	+	–
Sucrose	d	–	d	+	d	–
Mannitol	+	–	+	+	+	+
Inositol	–	–	d	d	–	d
Sorbitol	+	–	d	–	–	+
Rhamnose	+	–	d	+	d	+
Sucrose	d	–	d	+	d	–
Melibiose	–	–	d	+	d	d
Amygdalin	+	–	+	+	d	–
Arabinose	+	–	+	+	+	+

[a] Inactive species of *E. coli* may occur.
d = differs between strains; VP = Voges–Proskauer test.

Table 10.4 KCN-Resistant, Nonmotile Enterobacteriaceae. Found in Rodents and Rabbits

	Providencia	*Klebsiella*			
	rettgeri	*orthinolytica*	*oxytoca*	*planticola*	*pneumoniae*
KCN (growth)	+	+	+	+	+
Motility (37%)	–	–	–	–	–
β-Galactosidase	–	+	+	+	+
Arginine decarboxylase	–	–	–	–	–
Lysine decarboxylase	–	+	d	+	d
Ornithine decarboxylase	–	+	–	–	–
Citrate	d	+	+	+	d
H_2S	–	–	–	–	–
Urease	+	d	d	+	d
Tryptophane deaminase	+	–	–	–	
Indole	+	+	+	d	–
VP	–	d	d	+	d
Methyl red test	+	+	d	+	–
Gelatin liquefaction	–	–	–	–	–
Glucose (gas)	–	+	+	+	+
Lactose	d	+	+	+	+
Sucrose	–	+	+	+	+
Mannitol	d	+	+	+	+
Inositol	d	+	+	+	d
Sorbitol	–	+	+	+	+
Rhamnose	d	+	+	+	+
Sucrose	d	+	+	+	+
Melibiose	–	+	+	+	+
Amygdalin	d	+	+	+	+
Arabinose	–	+	+	+	+

d = differs between strains.

Table 10.5 KCN-Sensitive, Nonmotile Enterobacteriaceae. Found in Rodents and Rabbits

	Enterobacter agglomerans	*Yersinia pseudotuberculosis*
KCN (growth)	d	–
Motility (37%)	d	–
β-Galactosidase	+	d
Arginine decarboxylase	–	–
Lysine decarboxylase	–	–
Ornithine decarboxylase	–	–
Citrate	d	–
H_2S	–	–
Urease	d	+
Tryptophane deaminase	–	–
Indole	d	–
VP	d	–
Gelatin liquefaction	d	–
Glucose (gas)	+	–
Lactose	d	–
Sucrose	d	+
Mannitol	+	+
Inositol	d	–
Sorbitol	d	–
Rhamnose	d	+
Sucrose	d	–
Melibiose	d	d
Amygdalin	+	d
Arabinose	+	d

d = differs between strains.

with, *Salmonella*. Animal biosafety level 2 practices, containment equipment, and facilities are recommended for activities with experimentally or naturally infected animals.[20]

10.1.2 Pasteurellaceae

Pasteurellaceae are Gram-negative, mostly oxidase-positive, nonmotile, catalase-positive, pasteurella-form bacteria. No rodent species grow on MacConkey agar.

10.1.2.1 Impact on the host

Pasteurella multocida is a facultative pathogen of rabbits, and it may, in principle, infect rodents, but this is uncommon. It is mostly found in

conventional slaughter rabbits,[21,22] in which serotypes found are A, D, and F.[23] Infection is most often subclinical, and epizootic disease appears to be largely caused by environmental and host-related factors (e.g., environmental changes or experimental procedures). Respiratory disease occurs as "snuffles" (i.e., it is chronic) and in certain cases atrophic rhinitis,[24] otitis media,[25] and pneumonia, which may appear as fibrinopurulent pleuropneumonia or diffuse hemorrhagic pneumonia.[26] Respiratory problems occur most often in the spring and fall and are lowest in the summer. Conjunctivitis, abscesses, and acute septicemias have also been described, and the agent also may be found in the genitals. Direct contact with animals shedding *P. multocida* from nasal or vaginal secretions is considered the chief means of spread. The infection does not seem to spread between rabbits that are not in close contact with one another.[27,28] Transmission from other species (e.g., pigs and cattle) may occur.[29] A barrier system seems to be an efficient way of keeping rabbits free of the infection.[30]

Approximately 4–13% of barrier-protected rodent colonies are infected with *Pasteurella pneumotropica,*[31] which was first described by Jawetz in 1948.[32] Until then, the etiologic agent of *Pasteurella*-associated disease in rodents was diagnosed as *P. multocida*. Infections with *P. pneumotropica* are mostly latent. Brennan is often mentioned as the source for identification of this agent as a primary cause of pneumonia in rodents, but actually this study clearly revealed that pneumonia only appeared if the mice were also infected with *Mycoplasma.*[33] Later, it was linked to suppurative eye lesions in mice, but none of these mice were actually tested for *Mycoplasma* and viruses, and some mice examined did have the lesions without *Pasteurella* infection.[34] So, currently it should be generally accepted that *Pasteurella* as a pathogen in immunocompetent rodents is mainly secondary to a primary agent, such as *Mycoplasma pulmonis* or Sendai virus, although it has been described that it may alter cytokine expressions in C57BL/6 mice.[35] A few publications described *P. pneumotropica* as a primary pathogen causing abscesses, myopathy, and tumor regression in various immunocompromised animals, such as NOD/SCID (severe combined immunodeficiency) mice,[36] human immunodeficiency virus type 1 (HIV-1) transgenic mice,[37] and nude mice,[38] and it is also the experience that abscesses from mice in colonies not known to harbor virus or *Mycoplasma* infections often contain *P. pneumotropica,* as it has been described for years.[39] Furthermore, *P. pneumotropica* produces the toxins pnxIA and pnxIIA, which indicates virulence.[40] Transmission is mainly horizontal by droplets, but pseudovertical contamination may occur, as intrauterine infection may reach prevalences as high as 60% to 70%.[41] Therefore, puppies may be infected during birth or cesarean section. One should never introduce the uterus into the isolator during cesarean section. Rats and mice have their own species-specific subspecies of *P. pneumotropica,*[42] although cross infection should be considered possible.

Pasteurella pneumotropica does not spread rapidly to sentinels by either contact or soiled bedding; therefore, no matter whether cultivation or PCR is applied as a diagnostic tool, it should be applied directly to animals of the colony rather than to sentinels.[43]

Actinobacillus muris may be isolated from mouse colonies, where it has been claimed that it may cause infertility because of abortion, metritis, and stillbirths.[44] The infection rarely occurs, and when present, it is mostly latent. This rodent agent was formerly identified as *Pasteurella ureae* or *Actinobacillus urea*, which, however, seems to be a specific human agent.[45]

Haemophilus spp. are common findings in colonies of rats, mice, guinea pigs, and rabbits,[46] even if these animals are barrier bred,[47] and may also appear when analyzing the gut microbiota of mice by high-throughput sequencing.[48] One species from mice, *Haemophilus influenza-murium*, is clearly defined,[49] while for species isolated from rats, the nomenclature is developed, although some species can be clearly differentiated.[46,47,50] The pathogenicity is low, although infection in rats has been associated with mild inflammatory cell infiltration and a light diffuse hyperemia in the lungs.[51] Prevalence in infected colonies ranges from 50% to 100%,[47,51] and there seems to be some genetic background for a serologic response (e.g., BN rats are high, LEW rats are low, while BD IX, F344, and WKY rats are intermediate responders).[50] *Haemophilus* spp. have some species-specificity, but spread between species does occur; especially between rats and guinea pigs, to a lesser extent from rats to mice and apparently not that much from guinea pigs to mice.[52]

10.1.2.2 Characteristics of the agent

Pasteurella are small, coccoid, gram-negative rods (see Figure 3.2, item 8). They often occur as chains or pairs, and staining often is bipolar. *Haemophilus* may vary from coccobacilli to more rod-like cells. Colonies of *P. multocida* on blood agar are nonhemolytic and have a diameter of 1 to 3 mm after 24 h. Virulent strains are smooth or mucoid; rough colonies often indicate low virulence. Colonies of *P. pneumotropica* are small, 0.5 to 1 mm, white or yellow, with a certain smell (described by some as close to the smell of male mice, by others as the smell of ink or sperm). Samples should be inoculated directly on blood or chocolate agar. Chocolate agar should be used as the primary medium for the isolation of both *P. pneumotropica* and *A. muris*, although they will also grow on some types of blood agar. However, because there are certain types of blood that do not support growth, the safest procedure is to use chocolate agar (see Chapter 3). Growth of *Haemophilus* depends on X factor (protoporphyrin or protohemin), V factor (nicotine amide dinucleotide, NAD), or both; therefore, growth is most simply achieved by inoculation on chocolate agar to which lincomycin (5 µg/ml) or clindamycin (2 µg/ml) may be added to prevent growth of Gram-positive bacteria. Dependence on X or V factor is tested

using commercially available disks containing the factors, using one with the X factor, one with the V factor, and one with both on a Mueller Hinton agar (see Table C.7). Isolates growing close to only one of the single-factor disks *and* the X-V-factor disk are considered dependent on that factor; isolates growing only close to the disk containing both factors and eventually between the X-V-factor disks are considered dependent on both factors. For *Pasteurella*, cultivation for 24 h at 37°C will generally prove to be sufficient, but *Haemophilus* may need 48 h.

From diseased rabbits, the affected organ should be sampled directly for the isolation of *P. multocida*. In addition, blood may be sampled if septicemia is suspected. From healthy rabbits, the most appropriate sampling sites are the nose, trachea, lungs, and genitals. For *P. pneumotropica* and *Haemophilus*, the vagina or prepuce often seems to be the most successful site of isolation for these agents, which also may be isolated from nasoturbinates, conjunctiva, nasolacrimal duct, trachea, and lungs. *Pasteurella pneumotropica* may also be found in the uterus, and in older literature, it has been reported that during long periods, it may be latent in the intestines, from where it is difficult to isolate. Intestinal infection only seldom spreads to other organs.[53]

Biochemical characteristics for differentiating *P. multocida* from related species are given in Table 10.6. Three subspecies—subsp. *multocida*, subsp.

Table 10.6 Differentiation of Non-X- or V-Factor-Dependent Pasteurellaceae Found in Laboratory Rodents and Rabbits

	Pasteurella multocida	*"Pasteurella" pneumotropica*		*Actinobacillus muris*
		Jawetz	Heyl	
Lactose	−	+	+	−
Mannitol	+	−	−	+
D-Galactose	+	+	+	−
Raffinose	−	+	−	+
Esculin	−	−	−	+
Urease	−	+	+	−
Ornithine decarboxylase	+	+	+	−
Indole	d	+	+	−
L-Arabinose	d	−	+	−
Trehalose	d	+	+[a]	+
D-Mannose	+	+	+	+
Xylose	d	+	+	−
Melibiose	d	+	−	+

[a] Certain not clearly defined species from rats may be negative for trehalose.
d = differs between strains.

Table 10.7 Differing Subspecies of *Pasteurella multocida*

	Pasteurella multocida		
	subsp. ***multocida***	**subsp.** ***septica***	**subsp.** ***gallicidae***
Sorbitol	+	–	+
Dulcitol	–	–	+

Table 10.8 Differentiation of X- or V-Factor-Dependent Pasteurellaceae Found in Laboratory Rodents and Rabbits

	Haemophilus influenza-murium	Other spp.
X-factor dependent (hem)	+	–
V-factor dependent (NAD)	d	+
Esculin	–	–
Urease	–	–
Arabinose	–	–
Mannitol	–	d
Phosphatase	–	d
Indole	–	d
Xylose	–	d
Trehalose	+	d
Melibiose	–	d
Raffinose	–	d
Ribose	+	d

d = differs between strains.

septica, and subsp. *gallicidae*—have been defined. These differ as indicated in Table 10.7. *Pasteurella pneumotropica* may be divided biochemically into two biotypes, type Jawetz and type Heyl. Full identification may be performed. It should be noted that *P. pneumotropica* is a rather slow fermenter; therefore, long-term incubation (i.e., 48 h) may be necessary before safe conclusions should be drawn based on fermentation results. Some species specificity seems to exist between variants of *P. pneumotropica*. Mouse-associated strains are always trehalose positive; rat-associated strains are occasionally negative. For the test of biochemical characteristics of *Haemophilus*, the X and V factor must be added to the media. *Haemophilus influenza-murium* may be identified according to Table 10.8.

The commercial kit API 20NE (bioMérieux, France) may be used for identification of *P. multocida*, and it is also a commonly used diagnostic tool for diagnosis of *P. pneumotropica*. If applied, the basic rules for the use of commercial kits (Chapter 4) should be respected, and the slow reactions

of *P. pneumotropica* and the fact that *P. haemolytica* is not found in rodents should be kept in mind. Mannitol fermentation, ornithine decarboxylase, and indole reaction are traditionally used for differentiating between *P. pneumotropica* and *A. muris*. The latter is not included in the API system. The system seems to be most reliable for identification at the family rather than the genus level.[54] Identification tests may most easily be carried out using the commercial kit API NH (bioMérieux), although the computer software or the profiles supplied with the kit should not be expected to give any clear result for rodent species.

PCR directly on bacterial colonies or cultures represents an extremely rapid, sensitive method for identification of *P. multocida*[55] and for dividing these into different subtypes. PCR for diagnosis of colony infections of Pasteurellaceae have been around for 15 years,[56] and the future for screening in rodent colonies seems to be multiplex PCR assays, which are both less laborious and probably also more sensitive.[57] PCR and sequencing of either the 16S rRNA gene and other relevant target genes may also be applied for identification of rodent isolates.[58]

ELISA for antibodies may be used to diagnose infection with *P. multocida* in rabbit colonies, but the method of choice for full diagnosis is still considered cultivation and identification.[59] Also, in rodent colonies ELISA may be attempted for colony diagnosis of both Pasteurellaceae.[60,61]

10.1.3 Pseudomonadacea

10.1.3.1 Pseudomonas

10.1.3.1.1 Impact on the host *Pseudomonas* spp. are normal inhabitants of the environment and may contaminate tap water and even quaternary ammonium disinfectants.[62] They may be isolated from the respiratory, digestive, and genital systems of rats and mice. As several of those species isolated are just passive inhabitants, a variety of different *Pseudomonas* species may be observed. The most common is *P. aeruginosa*, which causes conjunctivitis, rhinitis, and pneumonia in immunodeficient, immunosuppressed, or stressed animals[63] and in transgenic mouse models of cystic fibrosis[64]; under such circumstances, the prevalence of diseased animals inside the colonies may reach 100%. The prevalence in infected colonies of immune-competent animals kept in a high-quality environment seldom reaches more than 5% to 10%, and in a Japanese survey, approximately 3% of facilities tested came out positive.[65] Poor hygienic conditions, especially in relation to water used for drinking and cleaning, may play an important role in the spread of *Pseudomonas* spp. *Pseudomonas fluorescens* and probably some other *Pseudomonas* spp. produce mucus in drinking nipples,[66] which, however, is not known to have any impact on the animals.

This condition is normally prevented by acidification of the drinking water with hydrochloric or citric acid or by chlorination.[66]

10.1.3.1.2 Characteristics of the agent *Pseudomonas* are Gram negative and appear as long, thin rods in young cultures and shorter rods (see Figure 3.2, item 7) in older cultures. Colonies usually spread on the agar surface and have a characteristic odor. As the culture becomes older, various types of pigmentation may occur. Cultivation of *P. aeruginosa* on King's agars (see Table C.18) at 35°C produces diffusable pigments. A variety of media may be used. Nonselective, nonindicative media such as blood or chocolate agar are useful. MacConkey agar may be used to favor selection. Propagation media may be used, but they are usually not needed because *Pseudomonas* spp. are easily isolated without propagation. Cultivation for 24 h at 37°C is sufficient. Cultivation at 42°C favors isolation of *P. aeruginosa*. Some species (e.g., *P. fluorescens*) may be favored by cultivation at temperatures as low as 4°C. *Pseudomonas* spp. may be isolated from any sampling site on the animal, but the skin and sites within the respiratory system are preferable as primary sampling sites. Rodent *Pseudomonas* spp. may be differentiated from other aerobic, Gram-negative, motile rods as being positive for arginine dihydrolase; the differentiation between different *Pseudomonas* spp. may be more difficult. Simple biochemical tests as proposed in Table 10.9 may be applied. The commercial kits API 20NE and API 20E (bioMérieux) clearly identify *P. aeruginosa* but are less reliable for other *Pseudomonas* spp. ELISA may be used to diagnose antibodies against *P. aeruginosa*.[67] PCR is an efficient tool for diagnosing the presence of *P. aeruginosa* in rodent colonies.[68]

10.1.4 Francisellaceae

10.1.4.1 Francisella

10.1.4.1.1 Impact on the host Only one species, *Francisella tularensis*, is to be considered in this genus. This is the causative agent of tularemia in humans and wild animals. As it is found in wild rodents and rabbits,[69] although not commonly,[70] it is in principle also of some importance in laboratory animal bacteriology, although it is seldom diagnosed in laboratory rodents and rabbits. One case in a hamster colony actually has been described.[71] The first sign was a ruffled hair coat, but within 48 h, the affected animals, which were 4 to 8 weeks old and weaned, were moribund, and the outbreak was connected with feeding fresh vegetables, which should be banned in today's laboratory animal colonies.[71]

10.1.4.1.2 Characteristics of the agent Successful isolation has been achieved from ulcers, lymph nodes, gastric washing, and sputum, but isolation is extremely difficult. For primary isolation, cystine glucose

Table 10.9 Differentiation of *Pseudomonas* spp. Found in Rodents and Rabbits

	Pseudomonas							*Brevendimonas*	
	aeruginosa	*fluorescens*	*putida*	*stutzeri*	*mendocina*	*pseudo-alcaligenes*	*alcaligenes*	*diminuta*	*vesicularis*
Oxidase	+	+	+	+	+	+	+	+	+
Growth at 42°C	+	–	–	+	+	d	d	–	–
Nitrate reduction	+	d	–	+	+	d	d	–	–
Esculin	–	–	–	–	–	–	–	–	+
Lecithin	–	+	–	–	–	–	–	–	–
Arginine dihydrolase	+	+	+	–	+	d	–	–	–
Gelatin liquefaction	+	d	–	–	–	–	–	d	d
Glucose	+	+	+	+	+	d	–	d	d
Lactose	–	d	d	–	–	–	–	–	–
Sucrose	–	d	d	–	–	–	–	–	–
Maltose	–	d	d	+	–	–	–	–	–
Mannitol	d	+	d	d	–	–	–	–	–

d = differs between strains.

blood agar may be used. Alternatively, chocolate agar supplemented with IsoVitale X (Becton Dickinson) and penicillin may be used. A heavy inoculum should be used, which may lead to visible colonies within 4 days. The organisms are small and pleomorphic. Colonies are up to 2 mm, blue-gray, round, smooth, and slightly mucoid. On cystine glucose blood agar, α-hemolysis is normally observed. Suspicious isolates are identified as *F. tularensis* by slide agglutination. Antibodies are commercially available (Quest Diagnostics, Madison, NJ, USA). Both ELISA and singleplex and multiplex PCR on blood have been described, but the sensitivity of cultivation still seems to be higher than any of these.[72]

The US CDC recommends biosafety level 2 practices, containment equipment, and facilities for activities with clinical materials of animal origin containing or potentially containing *F. tularensis*. Biosafety level 3 and animal biosafety level 3 practices, containment equipment, and facilities are recommended, respectively, for all manipulations of cultures and for experimental animal studies. An investigational live attenuated vaccine[10] is available. It is recommended for persons working with the agent or infected animals and for persons working in or entering the laboratory or animal room where cultures or infected animals are maintained.[20]

10.1.5 Aeromonadaceae, Xanthomonadaceae, Sphingomonadaceae, and Moraxcellaceae

The only genus in the Aeromonadaceae family to be isolated frequently from rodents is *Aeromonas*. Members can contaminate drinking water[73] and may be isolated from the cecum, genitals, and respiratory organs, but prevalences are low. They are often part of a mixed secondary flora in relation to wound infection, respiratory disease, and so on, but have little importance as primary pathogens, although experimental infection causes disease in BALB/c mice.[73]

They share some common characteristics with *Vibrio* spp.; therefore, differentiation in principle is necessary, although *Vibrio* is not a common finding in laboratory animals. This can be done by testing for sensitivity to O/129 (2,4-diamino-6,7-diisopropylpteridine), which is done by using a 150-μg disk in the agar diffusion inhibition assay (see Chapter 4). *Aeromonas* is resistant, and most *Vibrio* are sensitive.

Aeromonas found in rodents are *A. hydrophila*, *A. caviae*, or *A. veronii*, which may be differentiated according to Table 10.10. The commercial kit API 20NE (bioMérieux) is a helpful tool for identification on a genus level, but it is not usable for differentiation of species.

Xanthomonas maltophilia is occasionally isolated from rodents. It produces large pigmented colonies. A new name, *Stenotrophomonas maltophilia*, has been proposed. *Sphingomonas paucimobilis* (formerly *Pseudomonas*

Table 10.10 Differentiation of *Aeromonas* spp. Found in Rodents

	Aeromonas		
	caviae	*hydrophila*	*veronii* **subsp.** *sobria*
Gas from glucose	–	+	+
Esculin	–	–	+

paucimobilis) is occasionally isolated from the cecum of rodents. It is a long rod that forms large, yellow, butyrous, and sometimes mucoid colonies. *Acinetobacter* may occur as coccobacilli, coccoid rods, or more typical rods. Colonies are smooth, small, and nonpigmented. They are typically isolated from the respiratory system and genitals. *Acinetobacter junii*, which grows at 41°C, is most often isolated from guinea pigs; *Acinetobacter lwoffii*, which never grows at 41°C, is common in rats.

10.2 Betaproteobacteria

10.2.1 Alcaligenaceae

10.2.1.1 Bordetella

10.2.1.1.1 Impact on the host Only one species, *Bordetella bronchiseptica*, is of importance in laboratory animal bacteriology. It may be isolated from rabbits and guinea pigs and occasionally from rats; mice, hamsters, and gerbils are less prone to infection, and there seems to be some genotypic species specificity.[74] Disease, which is characterized by pneumonia, is most common in guinea pigs. In addition, pleuritis and pericarditis may be observed. The disease is often fatal, but whether this is mostly so if the animal is also infected with other respiratory pathogens, such as parainfluenza virus type III, is not fully understood. The agent is sensitive to erythromycin, gentamycin, kanamycin, chloramphenicol, and tetracycline,[75] of which only erythromycin seems to be safe in guinea pigs. In rabbits, disease is mainly subclinical and characterized by focal chronic interstitial pneumonia.[76] The young weanlings seem to be those who mostly drive spread of infection.[77] In wild rabbit populations, the prevalence, which is subject to seasonal variation, may reach more than 90%.[78] In rats, necrotizing bronchopneumonia may be observed. It may have a zoonotic potential in immunocompromised patients.[79]

10.2.1.1.2 Characteristics of the agent The cells of *B. bronchiseptica* are coccobacilli or short rods 0.2 to 0.5 µm wide and 0.5 to 2.0 µm long. The colonies are small and convex with an entire margin and may be β-hemolytic on blood agar. In contrast to human *Bordetella* spp., *B.*

bronchiseptica easily grows even on simple media (i.e., blood agar, chocolate agar, and MacConkey agar). Occasionally, lactose-sucrose agars are used indicatively, as the nonfermenting small colonies of *B. bronchiseptica* can easily be differentiated from colonies produced by fermenting bacteria. Aerobic cultivation for 24 to 48 h at 37°C is sufficient. The nose and trachea and, in affected animals, the lungs are suitable sampling sites.

Bordetella and *Brucella* are the only noncarbohydrate-utilizing, obligate aerobic, Gram-negative rods in laboratory animal bacteriology. *Bordetella bronchiseptica* differs from *Brucella* spp. by its ability to grow on MacConkey agar, which *Brucella* does not. This is not a major problem in the identification process because only *B. bronchiseptica* is a common finding in laboratory animal bacteriology. No carbohydrates are fermented. As no other members of *Bordetella* are to be expected in laboratory animals, differentiation between *B. bronchiseptica*, *B. pertussis, B. parapertussis,* and *B. avium* is not necessary. It is, however, easily done because *B. bronchiseptica* is motile, which *B. pertussis* and *B. parapertussis* are not, and *B. bronchiseptica* reduces nitrate, which none of the others do. Furthermore, *B. bronchiseptica* is urease positive. The commercial kit API 20NE (bioMérieux) easily identifies *B. bronchiseptica.*

PCR has been used with a high sensitivity and specificity for screening pig herds,[80] and there is no reason to assume that this should not also be applicable for guinea pig colonies, for example. ELISA can also be applied to screen for antibodies, probably only to a genus level, but as only *B. bronchiseptica* are found, this should be sufficient in most cases.[81]

10.2.2 Spirillaceae

10.2.2.1 Spirillum minus

Spirillum minus is a Gram-negative, motile, spiral bacterium 3 to 5 µm long that causes a slowly developing form of *rat bite fever* known as *Sodoku*, the symptoms of which are similar to rat bite fever caused by *Streptobacillus moniliformis.*[82] In older literature, several other names (e.g., *Spirochaeta morsis muris*, *Spirochaeta laverani*, *Spironema minor*, *Leptospira morsus minor*, *Spirochaeta muris*, and *Spirochaeta petit*) can be found, and although the name *S. minus* has been used for almost 100 years, it is not officially accepted because no reference strain is available.[82] *Spirillum minus* cannot be cultivated by any means, and as such, it is not possible to isolate it from rats, although it is obvious that rats may harbor this agent. Dark-field microscopy of blood or wound exudates from patients with a history of rat bites may reveal the presence of the spiral organisms. The disease has been reported primarily in Japan, but reports from the 1990s also located it in Kenya[83] and Brazil.[84] Gentamicin and penicillin both provide efficient treatment against this agent.[82]

10.3 Epsilonproteobacteria

10.3.1 Campylobacteraceae

10.3.1.1 Campylobacter

10.3.1.1.1 Impact on the host *Campylobacter coli* and *Campylobacter jejuni* have been isolated from the digestive system of mice, rats, hamsters, and rabbits.[85–87] Humans and some farm animal species are known to develop severe disease symptoms in relation to infection with *Campylobacter*, but this is not known to be the case in rodents and rabbits, and it is not clear whether *Campylobacter* infections in these species have a zoonotic potential. The prevalence rates as detected by cultivation of fecal samples from infected colonies are rather low, typically less than 5%, but PCR testing may reveal prevalence rates close to 100%.[85]

10.3.1.1.2 Characteristics of the agent *Campylobacter* spp. are arching, motile rods, often appearing as a comma or, if grouped in pairs, as a sea gull (see Figure 3.2, item 10). In older cultures, the cells may become coccoid. Colonies on TVP agar (see Table C.19) are small, either gray or transparent, and nonhemolytic. They may also appear as a thin film on the agar, however, in a thicker layer than the films created by *Helicobacter* spp. Feces and stool may be sampled from live animals. From euthanized animals, cecal scrapings, gallbladder (do note that the rat does *not* have a gallbladder), as well as intestinal contents may be cultivated as described for *Helicobacter* spp. (see the next section) using a 0.65-µm filter. TVP agar (see Table C.19) may be used. Propagation on commercially available enrichment broth is also a possibility.[88] Incubation must be microaerophilic for at least 48 h but preferably for 3 to 5 days at 37°C, either sealed with a microaerophilic gas kit or in a CO_2 incubator (see Chapter 3). The optimal atmosphere is 10% CO_2, 85% N_2, and 5% O_2. Identification is carried out according to Table 10.11.[89] Alternatively, commercial kits such as API Campy or the *Neisseria–Haemophilus* (NH) identification card/Vitek 2 (bioMérieux) are applicable, and they generally have high specificity but low sensitivity compared to matrix-assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).[90]

The US CDC recommends biosafety level 2 practices, containment equipment, and facilities for activities with cultures of, or clinical materials potentially infected with, *C. coli* and *C. jejuni*. Animal biosafety level 2 practices, containment equipment, and facilities are recommended for activities with naturally or experimentally infected animals.[20] Vaccines are not available for use in humans.

Table 10.11 Differentiation of Common *Helicobacter* and *Campylobacter* spp. from Rodents

	Helicobacter							*Campylobacter*	
	cinaedi	*bilis*	*hepaticus*	*muridarum*	*rodentium*	*rappini*	*trogontum*	*jejuni*	*coli*
Nitrate reduction	+	+	+	−	+	−	+	+	+
Alkaline phosphatase	−	?	?	+	−	−	−	d	d
Urease	−	+	+	+	−	+	+	−	−
Hippurate hydrolysis	−	−	−	−	−	−	−	+	−
γ-Glutamyl transferase	−	?	?	+	−	+	+	d	−
Growth at 42°C	−	+	−	−	+	+	+	+	+
Nalidixic acid (30 µg)	S	R	R	R	R	R	R	S	S
Cephalothin (30 µg)	I	R	R	R	R	R	R	R	R

Source: Some data from Fox JG, Lee A. *Lab Anim Sci* 1997; 47(3):222.

d = differs between strains; R = resistant; S = susceptible

10.3.2 Helicobacteraceae

10.3.2.1 Helicobacter

10.3.2.1.1 Impact on the host Several rodent *Helicobacter* species have been isolated (Table 10.12).[91–102] The primary colonization niches in rodents are the cecum and colon, with specific species capable of alternative colonization (Table 10.12).[91] *Helicobacter hepaticus, H. trogontum, H. bilis, H. typhlonius, H. rodentium, "H. rappini,"* and *H. ganmani* persistently colonize the lower bowel with shedding of the organisms in feces, promoting horizontal transmission through fecal-oral contact.[91] Clinical significance has only been documented in relation to a few of these. Some species are known to cause hepatitis and cancer (Table 10.12). Susceptibility to disease seems to be dependent on genetics; for example, A/JCr, SCID, and IL-10 knockout mice seem to be highly susceptible with macroscopically observable enlarged, edematous ceca and colons and eventually rectal prolapses and enlarged mesenteric lymph nodes,[103,104] while immunocompetent C57BL mice seem to be resistant.[105] In IL-10 knockout mice, the composition of the gut microbiota also seems important for severity grades.[106] In immunodeficient mice, the cecum and colon develop mononuclear inflammatory cell infiltrates in the mucosa and submucosa, and epithelial cell hyperplasia is often significant with frequent villous-to-papillomatous folds extending into the lumen and evidence of a high mitotic index in the crypts.[91] Goblet cells are variably fewer in number in the proliferative mucosa.[91] Liver lesions consist of multifocal-to-coalescing foci of coagulative hepatocyte necrosis around small intralobular hepatic venules, along with Kupffer, Ito, and oval cell hyperplasia.[107] Both wild and laboratory mice show prevalences of *Helicobacter* infection in the range 85–100%,[107,108] with *H. rodentium* the most common in wild mice.[107] It should be expected that approximately 15% of mouse research colonies in Europe and the United States are infected with some species of *Helicobacter*[31,109]; this is probably only the case for 5–10% of rat colonies.[31] Some rodent *Helicobacter* spp. may have some zoonotic potential.[110,111] In addition to the disturbance that the pathological changes may make in studies, the competition and coaction between spontaneous and experimental *Helicobacter* infection when rodents are used as models for human infection with *H. pylori* may be an important factor if experimental infection fails.[112] Vitamin D receptor knockout mice have increased abundance of Helicobacteraceae (and Proteobacteria in general) in their feces, but supplying them with the active form of vitamin D decreased severity of colitis induced by dextran sodium sulfate and reduced the numbers of Helicobacteraceae in the feces.[113]

10.3.2.1.2 Characteristics of the agent *Helicobacter* spp. are helical, curved, or straight Gram-negative rods that are approximately 0.3 to 1 µm

Table 10.12 *Helicobacter* spp. in Rodents and Their Clinical Importance

Name	Species	Location	First report	Clinical significance
H. cinaedi	Syrian hamsters	Cecum, colon[1]	1989[2]	Not reported
H. muridarum	Rats, mice	Cecum, colon, stomach[1]	1992[3]	Not reported
H. rappini	Mice	Cecum, colon[1]	1993[4]	Not reported
H. hepaticus	Mice, Mongolian gerbils	Liver, cecum, colon[1]	1994[5]	Typhlocolitis and hepatitis[6] in immunodeficient and specific immunocompetent mice; liver[7] and colon[8] cancer in specific strains
H. bilis	Mice, rats, Mongolian gerbils Syrian hamsters	Liver, cecum, colon[1]	1995[9]	Typhlocolitis[10] and hepatitis[11] in immunodeficient[12] and specific immunocompetent strains
H. trogontum	Rats	Cecum, colon[1]	1996[13]	Not reported
H. cholecystus	Syrian hamsters	Gall bladder[1]	1996[14]	Not reported
H. rodentium	Mice, rats	Cecum, colon[1]	1997[15]	Not reported
H. aurati	Syrian hamsters	Cecum, colon, stomach[1]	2001[16]	Gastritis and intestinal metaplasia[17]
H. mesocricetorum	Syrian hamsters	Cecum, colon[1]	2000[18]	Not reported
H. ganmani	Mice	Cecum, colon[1]	2001[19]	Not reported
H. typhlonius	Mice, rats	Cecum, colon[1]	2001[20]	Typhlocolitis in IL-10 knockout mice[21]
H. mastomyrinus	Mice, *Mastomys*	Cecum, colon [1]	2005[22]	Ulcerative typhlocolitis in telomerase-deficient mice[23]
H. magdeburgensis	Mice	Ileum, colon[24]	2010[24]	Not reported

Table 10.12 (continued) *Helicobacter* spp. in Rodents and Their Clinical Importance

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continued

Table 10.12 (continued) *Helicobacter* spp. in Rodents and Their Clinical Importance

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wide and 1 to 5 µm long (see Figure 3.2, item 6). Bacterial growth on an agar plate appears as a thin film, which may not be observed if the plates are not properly inspected. The optimal sampling sites in mice are the liver and the inner lining of the cecum and of course those sites listed in Table 10.12. From live mice, fecal pellets may be used. From the liver, a direct smear on the agar plate may be effective, while the cecum, stomach, and fecal pellets have to be sampled with great care. The inner lining is scraped with a sterile scalpel, and the scraping is then immersed in sterile 0.9% saline, which is whirl mixed. The immersion is then filtered directly on an agar plate or into an enrichment broth. The pore size of the filter should be 0.45 µm for *H. hepaticus* and 0.65 µm for *H. bilis, H. trogontum*, and *H. rappini*. Chocolate agar seems to support growth. From the cecum, it may be desirable to use a selective medium, such as TVP agar (see Table C.19), although it does not support growth as much as chocolate agar does. The medium is incubated for 3 to 5 days at 37°C, either sealed with a microaerophilic gas kit or in a CO_2 incubator (see Chapter 3). The optimal atmosphere is 5% CO_2, 90% N_2, and 5% H_2.

Broths to be used for tests for biochemical properties in *Helicobacter* spp. must be supplemented with 10% fetal calf serum. Growth is facilitated if the flask is placed in a water bath shaker at 150 rpm, fitted with a gassing hood connected to a microaerophilic gas mixture. The commercial kit API Campy (bioMérieux) may be helpful in the identification process, although the computerized identification procedure does not reveal rodent *Helicobacter* spp. Results from in-house tests as well as the commercial kit may be interpreted using Table 10.11.

ELISA may be used to detect antibodies against *H. hepaticus*. However, the sensitivity may be low and correlated to the degree of hepatitis. The

specificity also is not very high.[106] Males above the age of 12 months or females above the age of 18 months should be sampled.

PCR is currently the most rapid and perhaps the most sensitive method for diagnosis of *Helicobacter* spp., and it may be conducted on fecal pellets, cecal contents, or tissue homogenates.[114] A purification procedure is necessary to prevent inhibition.[91,115]

In human diagnostics, enzymatic assays are applied, and these may be applied for identification of rodent isolates, but because of extremely low sensitivity, they are not applicable as a diagnostic tool in rodent colonies.[116]

10.3.2.2 Rabbit-associated cilia-associated respiratory bacillus

Infection with CAR (cilia-associated respiratory) bacillus has been reported in Europe, the United States, and Japan in mice, rats, and rabbits and in farm animals such as pigs and goats. CAR bacillus isolates from, on the one hand, rats and mice; on the other hand, rabbits are host specific and should be regarded as being different bacteria that belong to distinct genera. Determination of gene sequences have shown that CAR bacillus from rats and from mice are related to *Flavobacterium*[117]; the rabbit isolates show a greater similarity with *Helicobacter.*[118] In rabbits, no clinical signs of respiratory disease have been observed, although histopathologic examination of the respiratory tree may reveal mild hyperplasia of lymphoid nodules subjacent to the respiratory mucosa with scattered CAR bacilli in the lower respiratory tract.[119] Contact infection may play a major role in the transmission of this disease.

Cultivation in traditional bacteriological media is difficult. For several years, propagation by inoculation of embryonated chicken eggs via the allantoic route has been used. The agent may also be propagated in Dulbecco's or Eagle's minimum essential medium supplemented with 10% fetal calf serum. In addition, 20% hamster tracheal organ culture soup may be added.[120]

10.4 Alphaproteobacteria

For Alphaproteobacteria, *Bartonella* spp. may be isolated from wild rodents,[121] and they can be cultivated on Columbia blood agar containing 10% whole fresh horse blood when incubated for up to 10 days at 30°C or 37°C in a moist atmosphere containing 5% CO_2.[122] However, they are not likely to be found in barrier-bred animals.

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chapter eleven

Actinobacteria

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11.1 Introduction

Actinobacteria are Gram-positive bacteria that are most frequently cultivable, although it may be more or less laborious. Although they are mostly considered as soil bacteria, they are standard members of the rodent gut,[1] dispersed on a range of different species; most of these species are not known to have any impact on the animal and therefore are not dealt with further in this text. Micrococcaceae exhibit some cross-identification issues with the pathogenic *Staphylococcus* spp., while Corynebacteriaceae, Actinomycetaceae, and Mycobacteriaceae contain pathogenic species; finally, Bifidobacteriaceae contain probiotic bacteria with a symbiotic impact on the animal, which may also make a difference in the animals when applied as animal models.

11.2 Micrococcaceae

Micrococcaceae in laboratory animals have previously been grouped together with the Firmicutes family Staphylococcaceae, which has mostly been based on morphology. Both grow easily on even simple media as

Table 11.1 Biochemical Characteristics of Micrococci Found in Rodents and Rabbits

	M. kristinae	*M. luteus*	*M. roseus*	*M. sedentarius*	*M. varians*
Colony pigmentation	Yellow	Yellow	Red	Cream	Yellow
VP	+	–	–	–	–
Glucose fermentation	+	–	d	–	+
Arginine dihydrolysis	–	–	–	+	–
Nitrate reduction	–	–	+	–	+

d = differs between strains; VP = Voges–Proskauer test.

pigmented colonies, at least 1 mm in size, and the cells are grouped as grapes (see Figure 3.2, item 1). Micrococcaceae have an oxidative utilization of carbohydrates—as determined by the OF (oxidative–fermentative) test—and do not grow under anaerobic conditions; staphylococci are fermentative in the OF test and do grow under anaerobic conditions. Staphylococcaceae grow in a 12% NaCl broth and are sensitive to lysostaphin, which is not the case for micrococci. The main differences are summarized in Table 8.1.

Micrococci are obligate aerobic cocci, which after 24 h of incubation on blood agar grow as large pigmented colonies. They utilize carbohydrates oxidatively, but in general, they utilize far fewer carbohydrates than staphylococci. In the microscope, they are grouped as pairs or grapes. They can be isolated from a wide range of animal species worldwide, but they are not known to have any serious impact on laboratory animals. Different *Micrococcus* species are identified based on the reactions given in Table 11.1. The use of the commercial kit API STAPH (bioMérieux, France) also leads to an identification of micrococci.

11.3 Corynebacteriaceae

Previously, the Corynebacteriaceae genus *Corynebacterium* contained bacteria that had a common characteristic in that they showed typical V forms (see Figure 3.2, item 4), several of which form so-called Chinese letters. Such bacteria are characterized as coryneforms. However, in other characteristics, the members of the traditional *Corynebacterium* genus differed greatly from one another. Recent systematics have defined corynebacteria as coryneform, facultatively anaerobic, catalase positive, nonmotile rods; former members not fulfilling these criteria have been placed in other genera.

11.3.1 Characteristics of infection

Corynebacteria are frequently isolated from laboratory rodents, but little is known about precisely which types infect which laboratory animals. Many isolates are typed as corynebacteria but often are not identified further as rodent corynebacteria are not well described. Some well-characterized *Corynebacterium* spp. described in this chapter are known to be isolated from laboratory rodents. As corynebacterial systematics have not been thoroughly studied in laboratory animals, other species, some of which are difficult to identify on a species level, may as well be isolated.

Corynebacterium kutscheri has been found worldwide, but during the last decades, it has become less common in laboratory animals because they are bred and kept in modern facilities. However, it can still be isolated from conventional rats[2] and mice[3]; in 2007 a report that an infant girl developed a wound infection with *C. kutscheri* after a rat bite also shows that the agent may be zoonotic.[4] The agent has also, but not quite rarely, been isolated from guinea pigs and hamsters.[5] In immunocompetent rats, the infection is mostly latent, but the agent may cause abscesses in the superficial tissues and pulmonary emboli[6]; embolization in the mouse affects joints, liver, and kidney.[7] Deficiency of interleukin (IL) 10 as in IL-10 knockout mice seems to help clearance and reduce the pathogenicity.[8] Coinfection with viruses does not seem to activate latent *C. kutscheri* infections[9]; genetics seems to be involved in resistance and susceptibility of both rats[10] and mice.[11] Therefore, mortality varies between infected colonies. The agent is most likely spread with feces.[12] Transplacental infection has been demonstrated experimentally.[13] Male ICR mice seem to be more susceptible than females.[14] Studies of experimental virus infection may fail because of infection with *C. kutscheri*.[9]

Nude mice occasionally suffer from a syndrome characterized by scaling and crusty skin from which coryneform bacteria are isolated. The most common agent is identified as *Corynebacterium bovis*.[15,16] It may be detected in immunocompetent mice and in the nasopharynx of humans.[17] Microisolation or filter-topped cages are not effective in maintaining mice *C. bovis* free, and airborne spread is not blocked by this[16,17]; however, hot tunnel washing is efficient against cage contamination.[17] Antibiotic prophylaxis and treatment of clinical disease in experimentally naïve mice is unrewarding, eradication of bacterial infection is difficult, and severe disease associated with *C. bovis* is likely multifactorial.[18]

Corynebacterium renale may cause urinary calculi in young rats.[19] *Corynebacterium mastitidis* may cause suppurative adenitis of the preputial glands in mice, although this condition has also been related to a range of other bacterial species.[20]

The number of animals in colonies infected with *C. kutscheri* may be less than 5%; skin problems in nude mouse colonies might involve more than 80% of the animals.

11.3.2 Characteristics of the agent

In the microscope, all corynebacteria may be found as V forms (see Figure 3.2, item 4), which may be arranged as typical "Chinese letters." Although they are all Gram positive, Gram staining may be variable, in particular for *C. kutscheri*. With *C. kutscheri,* metachromatic granules are observed inside the rods, and these granules seem to be Gram positive, even when the culture loses its Gram positivity. After 24 h at 37°C on blood agar, gray-yellow, smooth, nonhemolytic colonies of 0.5 to 1 mm develop; after longer incubation, these may grow to 1.5 to 2 mm. On blood tellurite agar, colonies are black. Colonies of *C. renale,* after incubation on blood agar at 37°C for 24 h, are round, smooth, white, and opaque. After longer incubation, they become dry and granular. The cells are typical Gram-positive coryneforms, often arranged in pallisades (i.e., many cells stacked closely together). Clubforms with metachromatic granules may occur. *Corynebacterium xerosis* and *Corynebacterium minutissimum* colonies on blood agar are 1 mm and yellow to tan after 24 h at 37°C, but after 48 to 72 h, they usually will enlarge to more than 2 mm. In the microscope, they are typical corynebacteria. *Corynebacterium bovis* after 48 h on blood agar at 37°C forms colonies that are nonhemolytic, white, smooth, and arching. Coccoid forms may occur. *Corynebacterium urealyticum* colonies are small and gray. Coccoid forms may occur. Most rodent species are nonhemolytic, although hemolytic strains of *C. kutscheri* have been described. They do not grow on MacConkey agar. Aerobic incubation at 37°C for 24 to 48 h is suitable.

The oral cavity, feces, cecum, genitals, and respiratory system are suitable sites for isolation of corynebacteria from healthy animals. Clinically affected animals should also be sampled from the cervical lymph nodes to search for *C. kutscheri* or from the renal pelvis to search for *C. renale*; attempts to isolate *C. bovis* should be performed on samples from the skin of affected animals.

All corynebacteria are Gram positive, nonmotile, fermentative and catalase negative, grow at 37°C, are negative in the Voges–Proskauer test, and do not produce H_2S. In general, corynebacteria produce acid from glucose. Rodent isolates of coryneform bacteria may be differentiated according to Table 11.2. The commercial kit API Coryne (bioMérieux) may be applied as well. Samples may be tested directly by polymerase chain reaction (PCR),[21] and antibodies may be found by enzyme-linked immunosorbent assay (ELISA).[22]

11.4 Actinomycetaceae

11.4.1 Actinomyces

Actinomyces may cause multiple pyogranulomatous inflammation with nodules in the skin, thorax, abdomen, and even the central nervous system.

Table 11.2 Differentiation of Aerobic and Facultatively Anaerobic Coryneform Bacteria from Laboratory Rodents and Rabbits

	Reduction of			Hydrolysis of		Acid from				
	Nitrate	Urease	β-Galactosidase	Gelatin	Esculin	Maltose	Sucrose	Mannitol	Xylose	Raffinose
Corynebacterium										
C. kutscheri	+	+	–	–	+	+	+	–	–	–
C. renale	–	+	–	–	–	+	d	–	d	–
C. bovis	–	d	+	–	–	–	–	–	–	–
Arcanobacterium										
A. haemolyticum	–	–	–	–	–	+	d	–	–	?

d = differs between strains.

Clinical signs may vary according to the affected site. Actinomycosis is rare in rodents today. Rabbits are less susceptible, but as laboratory rabbits are often bred and maintained under less-strict hygienic regimes than rodents, actinomycosis is most likely to be found in the rabbit, in which it is a chronic disease with clinical symptoms such as diarrhea and weight loss.

Members of the *Actinomyces* are branching, filamentous, Gram-positive rods. In principle, aerotolerant species occur, such as *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*), which causes pyogenic processes in farm animals. However, species isolated from rodents and rabbits show sparse, if any, growth under aerobic conditions; therefore, in laboratory animal bacteriology, *Actinomyces* may be regarded as an anaerobic genus, and incubation should be performed as such. Cultivation may be performed on anaerobic chocolate agar (see Table C.6) at 37°C. The inoculated plates are incubated for at least 48 h, inspected under a stereomicroscope, reincubated, and then inspected and reincubated every 3 to 5 days for up to 4 weeks. From affected animals, the affected organs should be sampled; in healthy animals, it is often most successful to isolate *Actinomyces* from the nose. *Actinomyces* spp. from laboratory animals have not been systematically defined. Species isolated from hamsters show greatest similarity with *Actinomyces bovis*, and species isolated from rabbits are more likely to show similarity with *Actinomyces israelii*. Neither is fully identical. They may be differentiated from one another on the basis of the morphology of the colonies. The microcolonies of *A. israelii* are filamentous, and those of *A. bovis* are smooth and nonfilamentous. Fully grown colonies of *A. israelii* are "molar-like"; that is, they are 1 to 2 mm and look like a tooth. Colonies of *A. bovis* have a sharp edge and are smooth. *Actinomyces israelii* is a more typical *Actinomyces* (i.e., filamentous and branching), and *A. bovis* may be coryneform. *Actinomyces israelii* occasionally grows aerobically, but *A. bovis* never does. Differentiation may be attempted using the kit API 20A (bioMérieux).

11.4.2 Arcanobacterium

Arcanobacterium spp. were formerly designated as *Corynebacterium* spp. *Arcanobacterium haemolyticum* has occasionally been isolated from the pharynx of various animal species and humans. Its occurrence and impact on rodents and rabbits have not been described in detail; there is a report that *Arcanobacterium pyogenes* may cause pneumonia in rabbits.[23] The morphology resembles that of corynebacteria, but it differs from these by being catalase negative. Differentiation from the catalase-negative regular rods may be based on simple morphological characteristics. Microscopy of *Arcanobacterium* shows rods, some of them in V forms. In older cultures, coccoid forms will appear. This is not likely to

be confused with *Erysipelothrix*, which are long filamentous rods, but it may be confused with lactobacilli. However, lactobacilli do not occur as V forms. Furthermore, prolonging the incubation to more than 24 h enlarges the small colonies of *Arcanobacterium*; the pinpoint colonies of *Lactobacillus* remain small on most substrates.

11.5 Mycobacteriaceae

11.5.1 Characteristics of infection

The Mycobacteriaceae genus *Mycobacterium* includes several species, most of which are saprophytic; a few are pathogenic for a range of animal species. Typically, the pathogenic mycobacteria cause granulomas with epithelioid cells in various organs (e.g., the lungs), a condition referred to as tuberculosis. In primates and ruminants, this is far more common and as such a far more important condition than in small laboratory animals such as rodents and rabbits. These species are to some extent prone to infection and disease, but spontaneous tuberculosis and other types of mycobacterial disease are not likely to become a problem in laboratory rodents and rabbits. Undefined *Mycobacterium* spp. can be identified in the gut of mice by both cultivation[24] and high-throughput sequencing,[1] however, with little knowledge whether it actually has any impact on the mice and their application as models. *Mycobacterium avium-intracellulare* once was reported as a spontaneous, latent infection of C57BL/6 mice in a colony, in which 63% of the mice had characteristic mycobacterial lung lesions (i.e., foci with macrophages and multinucleate giant cells).[25] *Mycobacterium lepraemurium* causes murine leprosy, characterized by granuloma formation around the veins and the capillaries containing so-called Lepra cells (i.e., large histiocytic cells with expanded pale cytoplasm and large pale nuclei, which are eccentrically situated). These cells fuse and congregate into nodules.[26] This, however, is a disease of wild rodents and has not been described in laboratory rodents. Infection occurs either aerogeneously or through contaminated diets.

11.5.2 Characteristics of the agent

Mycobacteria are gracile rods, typically 0.2 to 0.6 µm thick and 1 to 4 µm long. They may branch. The cell morphology varies and is not even typical within one species. Generally, they stain slowly, but when stained, the dyes cannot be evacuated and as such, they are Gram positive and acid fast. Colony morphology also is not uniform, but some characteristics are uniform within a species (Table 11.3). Some *Mycobacterium* spp. (e.g., *Mycobacterium lepraemurium* and *Mycobacterium microti*) cannot be cultivated easily, and diagnosis is made on the basis of showing the presence

Table 11.3 Characteristics of *Mycobacterium* spp. Found in Rodents and Rabbits

			Sensitivity to infection				
	Characteristics of infection	Colony morphology[a]	Rat	Mouse	Hamster	Guinea pig	Rabbit
M. tuberculosis	Generalized	Flat, rough, spreading to irregular periphery	–	–	++	++	+
M. bovis	Generalized	Small, thin, often nonpigmented					
		Raised, rough, later wrinkled and dry	–	–	+	++	++
M. avium/M. avium intracellulare	Mostly local in lymph nodes and lungs	Thin, transparent, glistening					
		Smooth, entirely rounded					
		Some colonies rough and wrinkled	–	+	+	+	+
M. lepraemurium	Granulomas around veins and capillaries	Not easily cultivated	+	+	–	–	+
M. microti	Local lesions	Not easily cultivated	–	–	–	+	+

[a] From Corper H, Uyei N. Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast nonpathogens on different mediums with their reaction to chemical reagents. *J Lab Clin Med* 1930; 15:348–369.

of the agent in characteristic lesions. Samples from healthy animals, as well as samples from affected animals in which contamination with other agents is suspected, should be pretreated to destroy the contamination. The most classical methods for this are the sulfuric and oxalic acid methods[27] (Table 11.4) or methods based on other salts, acids, or bases (e.g., chlorhexidine).[28] Samples should be inoculated on Löwenstein-Jensen slants[27,28] (see Table C.20). Cultivation for pathogenic *Mycobacterium* spp. should be incubated aerobically at 37°C for at least 7 weeks. Examination for growth must be performed weekly. Previously, identification on a species level was based on variations in the catalase test, growth on MacConkey agar, and inoculation in laboratory animals; today, the most valid methods for identification will be real-time PCR,[29] eventually combined with sequencing,[30] or high-performance liquid chromoatography (HPLC),[29] although the last seems to be more time consuming.

Table 11.4 Oxalic and Sulfuric Acid Methods for Decontaminating Samples Previous to Isolation Attempts for *Mycobacterium* spp.

Oxalic acid decontamination	Sulfuric acid decontamination
Materials	
5% oxalic acid	5% sulfuric acid
Sterile phosphate-buffered saline (PBS)	Sterile PBS
4% NaOH	10-ml centrifuge tube
Phenol red indicator	
50-ml centrifuge tube	
Method	
1. Add 5% oxalic acid to the sample (maximum 10 ml) in the ratio 1:1 in the centrifuge tube.	1. Add 5% sulfuric acid to the sample (3 ml) in the ratio 1:1 in the centrifuge tube.
2. Whirl mix and leave at room temperature for 30 min with occasional mixing.	2. Cap the tube and leave it at room temperature for 20 min at constant mixing.
3. Add PBS to 50 ml.	3. Add PBS ad 10 ml.
4. Cap the tube and mix it several times by inverting.	4. Cap the tube and mix it several times by inverting.
5. Centrifuge 15 min at 3000*g* or higher, decant the supernatant, and add a drop of phenol red to the sediment.	5. Centrifuge 15 minutes at 3000*g* or higher and decant the supernatant.
6. Neutralize with NaOH.	6. Wash the sediment several times in PBS.
7. Resuspend in PBS and use the suspension for inoculation.	7. Resuspend in PBS and use the suspension for inoculation.

From affected animals, the affected sites are sampled. From healthy animals, feces should be sampled.

The US Centers for Disease Control and Prevention (CDC) requires biosafety level 2 practices, containment equipment and facilities for preparation of acid-fast smears, and culturing of clinical specimens potentially infected with *M. tuberculosis* or *M. bovis*, provided that aerosol-generating manipulations of such specimens are conducted in a class I or II biological safety cabinet. The CDC requires biosafety level 3 practices, containment equipment and facilities for propagation and manipulation of cultures of *M. tuberculosis* or *M. bovis*, and for animal studies utilizing nonhuman primates experimentally or naturally infected with *M. tuberculosis* or *M. bovis*. Animal studies utilizing guinea pigs or mice can be conducted at animal biosafety level 3. Skin testing with purified protein derivative (PPD) of previously skin-tested-negative laboratory personnel can be used as a surveillance procedure. A licensed attenuated live vaccine (BCG) is available and is used in some countries, especially in Western Europe.[31]

11.6 *Bifidobacteriaceae*

The Bifidobacteriaceae *Bifidobacterium* spp., which until the 1950s were all designated as *Lactobacillus bifidus*, are among the major members of the colon microbiota of mammals, although their numbers in mice are much lower than in humans.[1] A high level of *Lactobacillus* spp. and *Bifidobacteria* is strongly correlated to low levels of inflammation in mice[32] and leptin in rats,[33] which also fits well with these bacteria acting protectively against inflammatory bowel disease (IBD) in both IL-10 knockout[34–37] and Smad3 knockout mice,[38] allergic sensitization in mice,[39,40] and myocardial infarction in rats.[33] Some *Bifidobacterium* spp. are used as probiotics for humans, with many of these species tested in mouse models. In some type 1 diabetes-prone nonobese diabetic (NOD) mice, they may not be found at all[1]; on the other hand, feeding a diabetes-promoting gluten-containing diet propagates *Bifidobacterium* spp.[41]

Bifidobacterium spp. are anaerobic and frequently branched bacteria. The simplest way to identify and quantify them is by quantitative PCR on fecal samples,[32] but they may be cultivated if diluted fecal samples are plated on MRS agar supplemented with 20 mg/l nalidixic acid, 8.5 mg/l polymyxin B, 50 mg/l kanamycin sulfate, 35 mg/l iodoacetic acid, and 25 mg/l 2,3,5-triphenyltetrazolium chloride, so-called bifidobacteria-selective *Bifidobacterium* iodoacetate medium (BIM) agar,[42] and incubated at 37°C for 5 days in an N_2-CO_2-H_2 (8:1:1) atmosphere.[40] The atmosphere may be varied systematically to be applied for identification, as different *Bifidobacterium* spp. are O_2 hypersensitive, O_2 sensitive, O_2 tolerant, or microaerophilic.

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chapter twelve

Spirochaetae

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12.1 Introduction

Spirochaetes are motile, spiral bacteria that are difficult to cultivate in ordinary media. They may be divided into three families: Spirochaetaceae, which contains the genus *Treponema*, of importance in rabbit bacteriology; Brachyspiraceae, which contains the genus *Brachyspira* of some importance in rodent bacteriology; and Leptospiraceae, which contains the genus *Leptospira*, of importance in rodent bacteriology.

12.2 Treponema

12.2.1 Impact on the host

More than 30 species of *Treponema* have been described. None of these are of any importance in rodents; one species, *T. paraluis cuniculi*, the causative organism of rabbit syphilis, must be considered in rabbits. Clinical syphilis in rabbits is a painful condition primarily localized to/around the genitals. Inflammation starts with development of edema in the vulva, vagina, or prepuce, which over some time develops into vesicles, which may turn into ulcers.[1,2] In severe and more chronic cases, erosions may appear in the head region as well. In contrast to human syphilis caused by *Treponema pallidum*, rabbits do not suffer from generalized cases involving the central nervous system. Spread is primarily venereal; therefore, the prevalence is highest in breeding colonies, where latent carriers may be found.[3] Infection with *T. paraluis cuniculi* may disturb the use of rabbits

in human syphilis research and diagnostic work. Today, most colonies of laboratory rabbits bred under acceptable hygienic conditions should be free of the organism, but in household rabbits, the disease may still be diagnosed.[2] In a study, the mean age of onset was 8.8 months, and treatment with oral administration of chloramphenicol or long-acting penicillin by intramuscular injection seemed to be successful.[2] The disease is likely to be maternally transmitted.[2]

12.2.2 Characteristics of the agent

Treponema paraluis cuniculi is approximately 0.18 µm wide and 6 to 15 µm long. It is regularly helical (see Figure 3.2, item 6). It cannot be seen in ordinary light microscopy without staining. Dark-field microscopy is the method of choice for observing motility in fresh preparations, but phase-contrast microscopy may also be used. Rapid rotation around the axis may be observed. The agent may also be observed in Giemsa, India ink (contrast), or immunofluorescense (IF) stains. The last is obviously helpful as a diagnostic tool, but of course, none of the three methods is able to show motility. To prepare a slide for microscopy, the crustae of an ulcer should be removed and the wound carefully cleaned with physiological saline. After drying the wound with gauze, some wound fluid is pressed out. A drop is mixed with physiological saline or tap water on a slide. Slides to be used for Giemsa or IF stains are air dried. Fixation and IF staining are then performed as described in Chapter 4. Slides for Giemsa staining are placed in methanol for 10 s and then in Giemsa solution (Merck, Germany) for 18 h. The staining solutions should be changed several times. For dark-field microscopy, a coverslip is placed on the immersion, and the preparation is microscoped within 20 min. First, observations are done through the ×400 magnification objective. When an organism has been localized, a drop of immersion oil is placed on the coverslip and motility is observed at ×1000 magnification.

Treponema paraluis cuniculi cannot be cultured using ordinary bacteriological media, probably because of rather specific demands on the atmosphere. Inoculation of specimens into noninfected rabbits has been used continuously for propagation. It has been shown that *T. pallidum* is microaerophilic rather than anaerobic, and that it can be propagated at least for some successive generations in complex cell media if the atmosphere contains only 1% to 5% O_2 and the medium contains reducing agents.[2] Such cultivation conditions do not seem to be of much use in routine diagnostic work.

Antibodies are easily diagnosed in sera from infected rabbits. As there are no serological differences, commercial kits for *T. pallidum* are fully usable for rabbits.[1] The method of choice is the immunofluorescence assay (IFA) (see Chapter 4). Antigen is commercially available. A

less-sensitive but occasionally more specific alternative is hemagglutination. Erythrocytes sensitized with antigen are agglutinated in a microtiter plate with U wells. Dilution series are made if the test is to be quantitative. The value of using nontreponemal tests—such as the cardiolipin test used for human patients—for screening healthy rabbits is not fully clarified. Enzyme-linked immunosorbent assays (ELISAs) for human diagnosis have been described, but as these produce a number of false positives, they are commonly combined with the use of Western blots[4] (see Chapter 4).

Polymerase chain reaction (PCR) is usable for direct detection of *T. pallidum* in experimentally infected rabbits,[5] and there is no reason to believe that such PCR should not also be applicable to diagnose *T. paraluis cuniculi* in rabbits.

12.3 Brachyspira

Species such as *Brachyspira hyodysenteriae, Brachyspira pilosicoli*, and *Brachyspira intermedia,* which are zoonotic organisms, are currently isolated from wild rats as well as wild mice.[6] *Brachyspira* spp. do not seem to be found when sequencing the gut microbiota of barrier-protected laboratory mice, although they may be found in the gut of healthy humans.[7] Different *Brachyspira* spp. have previously been indexed as both *Serpulina* and *Treponema*.

Brachyspira spp. may be cultured anaerobically on tryptose soy blood agar plates at 37°C to 38.5°C in an atmosphere of 5% CO_2 and 95% H_2, under which circumstances they grow slowly.[8] Identification is most safely performed with species-specific PCR.[9]

12.4 Leptospira

12.4.1 Impact on the host

The genus *Leptospira* consists of maybe more than 20 species. Leptospirosis is a zoonotic disease in humans and dogs caused only by *Leptospira interrogans*, of which at least 180 serotypes have been described, which are grouped in 23 serogroups. In humans, disease is characterized by high fever, occasionally by subserosal hemorrhage, and seldom by jaundice. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroups *icterohaemorrhagiae* and *ballum*, and mice are the maintenance hosts for serogroup *ballum*.[10] These are latent infections that are mostly found in wild animals, which carry the agent in their renal tubules, from where it is shed in the urine.[10,11] Rats experimentally infected with the serovar *icterohaemorrhagiae* are clinically asymptomatic and shed leptospires for up to 220 days.[12,13] Humans are infected after contact with such animals

or their urine or—more commonly—after contact with contaminated water or soil. The agents enter the patient through breaks in the skin or through the mucosal or conjunctival surfaces (e.g. when walking on bare feet,[14] doing gardening without gloves,[14] or handling conventional animals[15]). Laboratory rodents from properly protected and health-monitored colonies will not be harboring this agent.

12.4.2 Characteristics of the agent

Leptospira spp. are observed at best in dark-field or phase-contrast microscopy. The urine is the most obvious place to search for these agents in rodents. Before microscopy, the sample should be centrifuged at 1500*g* for 30 min. The supernatant is discharged, and a wet mount is prepared from the sediment. Alternatively, the kidneys may be crushed in a mortar and suspended 1:10 in phosphate-buffered saline (PBS). The suspension is centrifuged at 500*g* for 15 min, and the sediment is discharged; the supernatant is recentrifuged at 1500*g* for 30 min and prepared as for urine samples. In dark-field or phase-contrast microscopy, these motile organisms are helical rods, approximately 0.1 µm wide and 6 to 12 µm long (see Figure 3.2, item 6). The ends are hooked, and swellings are often observed. The movements are rotating and slowly gliding. For diagnosis, IF staining or ELISA[16] as well as macroscopic rapid agglutination[17] may be applied. The methods are performed as described in Chapter 4, but the slides for IF prepared with the sediment sampled as described previously should be fixed with absolute alcohol for 10 min, and a fluorescein-isothiocyanate-conjugated antileptospiral serum may be applied in a direct assay. The diluted antibody should be placed on the slide for 2 h at 37°C. For cattle urine, an IFA combined with immunomagnetic capture has shown high sensitivity,[18] and it is likely that this may be applied to rodents as well. As a simple method, Giemsa staining may be applied. Undiluted Giemsa solution (1 ml) (Merck) is added to 50 ml distilled water. Slides as fixed for IF staining are stained in the solution at 37°C for 8 h. Quantitative PCRs are sensitive and specific tools for routine diagnosis and differentiation and may detect as few as 10^2 *Leptospira* in clinical specimens, although probe mismatches do occur.[19]

One or two drops of urine or an aseptically prepared kidney suspension may be inoculated into a semisolid medium, such as the American Type Culture Collection (ATCC) 1470 modified *Leptospira* medium (http://www.atcc.org). Neomycin may be used to avoid overgrowth in urine cultivation. A series of tubes with 5 ml of medium are inoculated, each with a drop of urine. Two tubes are inoculated with undiluted urine, two tubes with a 1:10 dilution in PBS, two tubes with a 1:100 dilution, and two tubes with a 1:1000 dilution. A 30-µg neomycin antibiogram disk is added to each tube. For examination of the kidney, a piece of the kidney

is placed in a 5-ml syringe and crushed with the plunger so a few drops can be inoculated into the medium. The inoculated cultures are kept at room temperature. During the first week, nothing is done, but then a drop is examined by dark-field microscopy every week for 5 weeks and then twice per month for 4 months.

The US Centers for Disease Control and Prevention (CDC) recommend biosafety level 2 practices for all activities involving the use or manipulation of tissues, body fluids, and cultures known or potentially infected with *L. interrogans* and for the housing of infected animals. Gloves are recommended for the handling and necropsy of infected animals and when there is the likelihood of direct skin contact with infectious materials. Vaccines are not available for use in humans.[20]

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chapter thirteen

Tenericutes

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13.1 Introduction

In 1984, Tenericutes was validated as a bacterial phylum[1] and described by the lack of a cell wall leading to staining as Gram negative. Tenericutes includes one class, Mollicutes, of which the genera *Mycoplasma*, *Acholeplasma*, and *Anaeroplasma* may be more or less important in laboratory animal bacteriology. Rodents and rabbits may harbor *Mycoplasma*, and guinea pigs and rabbits may harbor *Acholeplasma*. *Anaeroplasma* are anaerobic Mycoplasmatales mostly described as a finding in the rumen of ruminants,[2] but high-throughput sequencing-based studies have shown a presence in the gut of mice.[3] However, little is known to which extent it has any symbiotic or pathogenic impact on the host and whether it cross-reacts with *Mycoplasma* spp. *Mycoplasma* and *Acholeplasma* differ simply in their demands for cholesterol in the medium: *Acholeplasma* grows on cholesterol-free media, and *Mycoplasma* does not. *Mycoplasma* spp. have previously been known under the name pleuropneumonia-like organisms.

13.2 Mycoplasma

13.2.1 Impact on the host

Mycoplasma pulmonis is the *Mycoplasma* most frequently encountered in laboratory animal bacteriology. In most individual cases, the sole presence of this agent in the host causes either mild symptoms or no symptoms at all. However, when complicated with other infectious agents, such as *Pasteurella pneumotropica*[4] or various viruses,[5] as well as environmental inducers such as raised ammonia levels,[6] disease symptoms may become more severe. It has been claimed that it also exacerbates symptoms of

infection with cilia-associated respiratory (CAR) bacillus, but this does not seem to be the case for all strains of *M. pulmonis*.[7] *Mycoplasma pulmonis* is more frequently found in rats than in mice.[8] The fraction of laboratory rodent colonies infected currently is less than 1%,[8–10] but 90–100% of pet rats are seropositive,[11] which indicates a risk also for laboratory rodents. In rats, it causes snuffles, ruffled hair coat, bronchopneumonia, and arthritis, mostly in a mild form. In addition, it colonizes genitals of both males and females,[12] and at least in the latter, it may affect reproduction. Even in the absence of clinical symptoms, *M. pulmonis* may be hazardous to experiments. It may raise the incidence of respiratory tract tumors,[13] decrease the cellular and humoral immune response,[14] decrease the severity of adjuvant arthritis,[15] and reduce the incidence of diabetes mellitus type 1 in biobreeding rats. In mice, clinical symptoms are similar to rats,[16] and in C57BL/6 mice, it may raise the proinflammatory cytokine level and enhance the metastatic capability of melanoma cells.[17] *Mycoplasma pulmonis* may colonize humans in contact with infected animals, which probably does not cause disease but is important for the spread of the agent.[18]

Mycoplasma arthritidis may cause arthritis in rats and mice, at least when used experimentally as a model.[19] It may, however, also occur as a spontaneous infection.[20] Infection is mostly asymptomatic,[20,21] but immunodeficiency, low social rank,[22] and genetics[23] may act as determinants of disease. In mice, it may also cause conjunctivitis and uveitis.[24] The last conditions are under the influence of the major histocompatibility complex.[25] It may cause various types of decreased cellular immune responses.[26,27]

Mycoplasma neurolyticum may be isolated from the conjunctiva and nasopharynx of rats and mice, in which it probably does not cause any symptoms, although some early descriptions indicate that it may cause conjunctivitis,[28] and even older papers report it as a contaminant that causes "rolling disease" in mice used for passage of cells, which happen to be contaminated with *M. neurolyticum*.[29] Spontaneous infections are not known to occur as a major problem in commercially bred laboratory rats and mice today.

Mycoplasma muris has been isolated from the vagina of mice.[30] It has only been isolated once in one colony. As it does not grow on ordinary *Mycoplasma* media, it is difficult to know whether isolation is uncommon because infection is uncommon or because isolation seldom is attempted. The impact on the mice is unknown. *Mycoplasma collis* has previously been isolated from the conjunctivas and nasopharynges of rats and mice,[31] but as with *M. muris*, this has not later seemed to be of major relevance.

Guinea pigs may harbor *Mycoplasma caviae,* which is apathogenic for guinea pigs.[32] The original isolating scientist by accident inoculated this agent into his own thumb and thereby developed a severe local infection.[33] It is, however, difficult from such case stories to judge the zoonotic potential of the organism. *Mycoplasma cavipharyngis* is another probably

apathogenic *Mycoplasma* found in guinea pigs.[34] Modern sequencing has shown that it is closely related to *Hemobartonella* and *Epirythrozoon*.[35] *Mycoplasma cricetuli*[36] and *Mycoplasma oxoniensis*[37] have been isolated from the conjunctiva of hamsters. The pathogenicity of these organisms seems to be rather low, and it does not seem to be a frequently occurring event.

Various types of rodent *Mycoplasma* spp. are likely to infect cell cultures, such as murine leukemia cell lines, and cell lines used for propagation of other infectious agents.[38]

13.2.2 Characteristics of the agent

Mycoplasmas are clearly Gram negative because they have no cell wall, but because they are not easily stained by Gram staining, other methods should be applied. Smears may be prepared for staining by mounting a punched-out colony on a slide with the growing side on the glass, holding it tilted and melting the agar onto the slide in a water bath, air drying, and fixating with Bouin's solution for 30 min. Such slides may be Giemsa stained. However, the method of choice for studying the cellular morphology seems to be dark-field or phase-contrast microscopy on nonfixed aqueous immersions. This will also allow demonstration of motility. Immunofluorescence staining directly on colony smears is also an option if the identity is known well enough to choose an antibody for staining.

The cellular morphology of mycoplasmas is rather variable, ranging from round to filamentous. The general principle is that young cultures in their logarithmic growth phase contain numerous filaments, and older cultures contain more coccoid forms. *Mycoplasma pulmonis* is usually spherical to pear shaped and has a diameter of 0.3 to 0.8 µm. Filaments may occur. *Mycoplasma neurolyticum* has the same shape as *M. pulmonis* and may show filaments up to 160 µm long. *Mycoplasma arthritidis* is pleomorphic, and smaller filaments may occur.

To be able to observe colony morphology, agar plates should be inspected under a binary microscope. Colonies may vary in size from 15 to more than 300 µm. Typical *Mycoplasma* colonies are said to have a fried-egg appearance (i.e., large colonies with a top in the center), but this is not necessarily a common characteristic for rodent myoplasmas. *Mycoplasma arthritidis* usually produces such colonies; *M. pulmonis* and *M. neurolyticum* do not. *Mycoplasma pulmonis* grows with large and raised colonies with a granulated or vacuolated surface. Other *Mycoplasma* spp. grow with multiple, hardly visible, colonies spread all over the agar surface.

Isolation may be attempted in broth as well as in solid medium. In general, media for *Mycoplasma*, in addition to the basic substances, contain yeast extract, horse serum, and penicillin. If *M. neurolyticum* is to be isolated, penicillin should be omitted because this agent is inhibited by the addition of penicillin. Horse serum should be inactivated by heating to

56°C for 30 min before adding to the medium. Various commercial media, such as BBLO PPLO™ agar and broth (BD Biosciences) or SP4 agar or broth (Hardy Diagnostics) are available. Both solid media and broth should be heavily inoculated (e.g., with minced tissue) if possible. It should, however, be noted that in glucose-containing broths, the tissue may induce a yellow change, which is not related to glucose metabolism. Ordinary media do not support growth of *M. muris*, which should be grown in the SP4 broth or on the SP4 agar. To detect growth, phenol red should be added to broths. For those mycoplasmas that ferment glucose, 1% glucose should be added; for those mycoplasmas that utilize arginine, 1% arginine should be added. The inoculated media are incubated at 37°C, preferably with 5% CO_2, as described for microaerophilic incubation (see Chapter 3), for example. In addition, a humid atmosphere should be used for cultivation on solid media. Growth will usually be achieved in 6 to 10 days, but occasionally, solid media have to be incubated for up to 3 weeks. Broths should not be incubated more than 1 week, after which they should be plated on a solid medium.

Some optimal sampling sites for various mycoplasmas are listed in Tables 13.1 and 13.2.

The ability to adsorb erythrocytes is characteristic for some *Mycoplasma* spp. (Table 13.1). Guinea pig erythrocytes should be used as a standard, but the species from which the isolate originated should also be used, possibly along with erythrocytes from a third species. In many laboratories, identification mostly has been based on immunological assays using either monoclonal- or polyclonal-specific antibodies. A classical method is inhibition of growth on an agar plate by placing in the middle of the plate a paper disk soaked in a dilution of a high-titer antiserum.[39] The dilution to be used must be found in pretesting with a sample of known identity. Immunofluorescence staining, dot blotting, and Western immunoblotting using a range of specific antimycoplasmal or antiacholeplasmal sera for identification may be performed as described in Chapter 4. Blotting may detect approximately down to 10^4 colony-forming units per milliliter depending on *Mycoplasma* species.[40] Cross-reaction between closely related *Mycoplasma* spp. (e.g., *M. pulmonis* and *M. arthritidis*) may be problematic in all immunological *Mycoplasma* identification assays depending on the nature of the antibody used.

All rodent mycoplasmas have been sequenced. Polymerase chain reaction (PCR) (see Chapter 5) is an optimal way of identifying mycoplasmas. Highly sensitive quantitative real-time PCR (qPCR) assays have been developed in which the nucleic acid content equivalent to (in principle) a single organism may be detected. Primers species specific for *M. pulmonis, M. arthritidis, M. neurolyticum, M. muris,* and *M. collis* may be selected; genus-specific primers may react with all *Mycoplasma* species as well as with members of the genera *Ureaplasma, Spiroplasma,* and *Acholeplasma.*

Table 13.1 Characteristics and Sampling Sites for *Mycoplasma* spp. Found in Mice and Rats

Mycoplasma	Species	Sampling site	Growth on serum yeast *Mycoplasma* agar	Penicillin resistant	Glucose catabolism	Utilizes arginine	Hemadsorption
M. pulmonis	Mice, rats	Respiratory system, genitals, joints	+	+	+	–	d
M. neurolyticum	Mice, rats	Conjunctiva, nasopharynx	+	–	+	–	–
M. muris	Mice	Vagina	–	+	–	+	+
M. arthritidis	Mice, rats, rabbits	Joints, conjuctiva, skin	+	+	–	+	–
M. collis	Mice, rats	Conjunctiva, Harderian gland, nasopharynx	+	+	+	–	?

d = differs between strains.

Table 13.2 *Mycoplasma* spp. Found in Rabbits, Guinea Pigs, and Hamsters

Mycoplasma	Species	Sampling site	Glucose catabolism	Utilizes arginine	Hemadsorption
M. caviae	Guinea pigs	Nasopharynx, vagina	+	d	–
M. cavipharyngis	Guinea pigs	Nasopharynx	+	–	+
M. cricetuli	Hamsters	Conjunctiva	+	–	–
M. oxoniensis	Hamsters	Conjunctiva	+	–	–

d = differs between strains.

PCR may also be used directly to detect *Mycoplasma* spp. in tissue samples. Tissue samples are frozen in liquid nitrogen. Approximately 1 g of the frozen tissue is ground into a fine powder and placed in a sterile glass with 1 ml of 0.1M NaCl, 0.1M Tris-HCl, and 1% sodium dodecyl sulfate (SDS) (pH 8.0); boiled for 10 min; and spun at 10,000*g* for 3 min. The supernatant is used for qPCR. As little as 1 pg of nucleic acid may be detected.[41]

Serology has been performed for several years to detect infection with *M. pulmonis* in rodent colonies. Since the 1980s, the method of choice has been either immunofluorescence assay[42] or enzyme-linked immunosorbent assay (ELISA)[43] (see Chapter 4), but multiplex, microbead, fluorescence techniques (Luminex Corp.) may be applied for simultaneous detection of antibodies to a range of murine agents, including *Mycoplasma*.[44] ELISA has also been widely used for detection of antibodies against *M. arthritidis.* These two mycoplasmas generally cross-react depending on the test applied.[45] If both agents are to be included in a health-monitoring profile, specific ELISA assays with both *M. pulmonis* and *M. arthritidis* antigen should be run to secure high sensitivity for both infections. Tests for immunoglobulin (Ig) G are more sensitive than tests for IgM, but generally, serological assays are fully reliable; for spontaneous infections, they seem to be more sensitive than cultivation methods.[8,46] *Mycoplasma* does not spread rapidly to sentinels.[47]

13.3 Acholeplasma

Guinea pigs and rabbits may harbor various *Acholeplasma* species. These organisms do not require cholesterol in the medium. They may grow aerobically, glucose is metabolized by some, and arginine and urea are not hydrolyzed. In guinea pigs, at least *Acholeplasma cavigenitalium*[48] and *Acholeplasma laidlawii*[49] have been identified. Spontaneous antibodies to *A. laidlawii* membrane lipids may be found in guinea pig sera.[50] *Acholeplasma laidlawii*[51] and *Acholeplasma multilocale*[52] have been isolated from the feces of rabbits. The pathogenicity of these organisms seems to be rather low. Cultivation and identification of these agents are similar to methods described for mycoplasmas. Optimal sampling sites may be found in Table 13.3.

Table 13.3 *Acholeplasma* spp. Found in Rabbits and Guinea Pigs

Acholeplasma	Species	Isolation site
A. cavigenitalium	Guinea pigs	Vagina
A. laidlawii	Rabbits, guinea pigs	Feces, vagina, nasopharynx
A. multilocale	Rabbits	Feces

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chapter fourteen

Verrucomicrobia, Deferribacteres, Fusobacterium, and TM7

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14.1 Verrucomicrobia

14.1.1 Akkermansia

14.1.1.1 Impact on the host

The phylum Verrucomicrobia is ubiquitous in soil and water, where members play important ecological roles.[1,2] *Akkermansia* is the only genus of the phylum Verrucomicrobia that to date has been found to play a role in the mammalian gastrointestinal tract. *Akkermansia muciniphila* is the only validly described member of the genus,[3] although at least eight different species exist within the *Akkermansia* genus, as determined by mining gut microbiome data sets for *Akkermansia*-like sequences.[4]

The Gram-negative, strictly anaerobic *A. muciniphila* is a relatively prominent member of the human gut microbiota, where it constitutes 0.1–3% of the total bacterial gut flora. As the name indicates, it is

closely associated with the gut mucus layer, where it feeds on mucin.[5] Monocolonization of germ-free mice with *A. muciniphila* has shown that it influences mucosal immune response[6]; in several studies, *Akkermansia* has been found to be negatively correlated with diseases such as type 1 diabetes (mice and humans), ulcerative colitis (humans), and autism (children) and seems to offer protection against metabolic disorders associated with diet-induced obesity (mice).[7–11] In contrast it seems to be positively correlated to the development of colon cancer in mice induced with azoxymethan.[12] Treatment with vancomycin has been found to propagate *A. muciniphila* in the gut of mice, leading to a gut microbiota dominated by this bacterium.[8] Also, feeding with xylooligosaccharides has been found to propagate *A. muciniphila* in mice.[13]

14.1.1.2 Characteristics of the agent

From complex environments (the gut), *Akkermansia muciniphila* can be cultivated using mucin medium, with mucin as a carbon source,[14] which gives the mucin-degrading *A. mucinihila* a competitive advantage. For pure cultures, for example, brain-heart infusion (BHI) can be used. Incubate at 37°C under anaerobic conditions. For enumeration, assays based on quantitative real-time PCR (qPCR) have been developed (e.g., see Hansen et al.[8]). Arnds et al.[15] described the development of a 16S ribosomal RNA (rRNA) gene-targeted Verrucomicrobia-specific probe suitable for studies based on fluorescence in situ hybridization (FISH) of *Akkermansia* and other Verrucomicrobia in tissue samples, for example.

14.2 Deferribacteres

Deferribacteres contains one order, Deferribacterales, which contains one family, Deferribacteraceae, which contains the genera *Calditerrivibrio, Denitrovibrio*, *Deferribacter*, *Flexistipes, Geovibrio*, and *Mucispirillum*. Comparisons of completely sequenced bacterial genomes show that Deferribacteres is phylogenetically proximal to the Proteobacteria and Nitrospirae.[16] Most of them are environmental, some of them have been found in the human oral cavity, but the only one of importance in laboratory animal bacteriology is *Mucispirillum schaedleri*.

14.2.1 Mucispirillum schaedleri

14.2.1.1 Impact on the host

Mucispirillum schaedleri is an obligate anaerobic spiral bacteria that, in the same way as *Helicobacter* and *Campylobacter*, colonizes the inner lining of the murine gut, but it has no close phylogenetic relationship to these genera.[17] It was first isolated by Schaedler and used as a member of the altered

Schaedler flora.[18] It was given the name ASF 457, and later, it was determined by sequencing gut bacteria of mice from some Australian facilities that it was a Deferribacteres species, and it was named *M. schaedleri*.[17,19] It still seems to be the only species of the phylum Deferribacteres found in the gut of mice.[20] The abundance is around 1%.[20] It is not found in the human gut,[20] but it has been isolated from rats.[17] It is primarily found in the ileum and proximal colon and at lower abundance in the upper small intestine and lower colon. In one study, it was not found in the cecum[21]; the cecum proved to be the most common site for isolation in another study.[17] When sequencing, it is found both in cecum and feces from mice.[20]

Little is known concerning its impact on the murine host. Mice that by knockout are made unable to produce the active form of vitamin D or the vitamin D receptor have in their feces, compared with wild-type mice, more bacteria from the Bacteroidetes and Proteobacteria phyla and fewer bacteria from the Firmicutes and Deferribacteres phyla.[22] Deferribacteres in the murine gut are known to be all *M. schaedleri*. A high level of *M. schaedleri* in the gut correlates to low levels of butyric and acetic acid in the cecum in a mouse model of autism induced with in utero exposure to valproic acid.[23]

14.2.1.2 Characteristics of the agent

When observed in wet mounts under phase-contrast microscopy, *M. schaedleri* have a thin, tight spiral morphology (see Figure 3.2, item 6), and the organisms are Gram negative.[17] Electron microscopic examination of a number of isolates revealed the presence of single, bipolar, unsheathed flagella.[17] In general, species within Deferribacteres can be cultivated anaerobically in Schaedler broth or blood-enriched anaerobic agar.[21] For the isolation of *M. schaedleri*, mouse intestinal mucus scrapings can be inoculated directly onto BHI agar, or they can be inoculated onto 0.65-µm filters (Millipore) on the agar surface and placed in a 37°C incubator (10% CO_2 and 95% humidity) for 2 h, after which the filters are removed.[17] They do not grow under microaerobic conditions, and all plates should be incubated anaerobically with a gas-generating kit at 37°C for 72–96 h.[17] Colonies are nonpigmented and nonhemolytic.[17] Table 14.1 shows some biochemical reactions.[17] They can also easily be screened for and identified by qPCR.[21]

14.3 Fusobacteria

14.3.1 Fusobacterium necrophorum

14.3.1.1 Impact on the host

In the host context, *Fusobacterium* is of importance only in the rabbit.[24] *Fusobacterium necrophorum* is not the only species within this genus, but it

Table 14.1 Biochemical Reactions for *Mucispirillum schaedleri*

Aerobic growth	–
Anaerobic growth	+
Motility	+
Catalase	+
Oxidase	+
Triphenyl-tetrazolium chloride reduction	–
Hydrogen-disulphide production in triple-sugar-iron agar	–
NO_3 reduction	–
Selenite reduction	–
Growth with	
Cephalothin (32 mg/l)	+
Carbenicillin (32 mg/l)	+
Cefoperazone (64 mg/l)	+
Tyrosine	–
Casein	–
Lecithin	–
Basic fuchsin (0.005%)	–
Methyl orange (0.032%)	–
Nalidixic acid (32 mg/l)	–
5-Fluorouracil (100 IU)	–
Sodium fluoride (0.05%)	–
NaCl (2–4%)	–
Potassium permanganate (0.1%)	–
Sodium arsenite (0.0001%)	–
Safranin (0.02–0.05%)	–
Crystal violet (0.0005%)	–
Janus green (1.01%)	–
Pyronin (0.02%)	–

is the main pathogen. In rabbits, *F. necrophorum* may produce either a phlemognous and purulent necrosis, typically starting in the nasal region, or a chronic suppuration over the legs and flank. Such clinical symptoms are not common in laboratory rabbits today, but as routine screenings for this agent are seldom performed in rabbit colonies, little is known about whether laboratory rabbits actually still harbor this organism. Whether laboratory animals also harbor some of the other species in the genus is not known.

14.3.1.2 Characteristics of the agent

Bacterial morphology is pleomorphic. Coccoid as well as longer filamentous rods may be observed. Metachromatic granules may be observed.

Colonies are white, small, and round. If human or rabbit blood is used, colonies on blood agar are hemolytic.

Chocolate agar with the addition of vitamin K and cysteine (see Table C.6) should be used for primary isolation. The inoculated medium is incubated anaerobically at 37°C for 24 to 48 h. Pure cultures may be grown on blood agar containing rabbit or human blood. Sampling is performed from the deep parts of the necrotic area in affected rabbits. From healthy animals, scrapings of the inner lining of the cecal mucosa should be used.

Fusobacterium spp. stain Gram negative and are characterized by resistance to vancomycin but sensitivity to both kanamycin and colistin. *Fusobacterium necrophorum*, in contrast to other *Fusobacterium* spp., will not grow in 20% bile, and it is indole and alkaline phosphatase positive. *Fusobacterium necrophorum* may be further divided into biovars: biovar A/F, also called *F. necrophorum* subsp. *necrophorum,* produces hemagglutinin and lipase, which biovar B, also called *F. necrophorum* subsp. *funduliforme,* does not.

14.3.2 Streptobacillus moniliformis

14.3.2.1 Impact on the host

Streptobacillus moniliformis may be isolated from mice, rats, and guinea pigs, and it is transmissible to humans, in which it causes *rat bite fever*, a purulent wound infection developing into petechial exanthema, polyarthritis, and fever and in some cases death if untreated.[25] Currently, it is not a common zoonotic condition in laboratory animal facilities, but fatal cases have occurred in the Western world after contact with wild rats. It has also recently been isolated from barrier-maintained mice,[26] but this is to be regarded as the exception rather than the rule. Also, *Spirillum minus* (Chapter 10) may be the cause of rat bite fever.

The laboratory animal most susceptible to the development of disease is the mouse, in which cases begin as swelling of the cervical lymph nodes and may turn into fatal septicemia. Chronic cases are characterized by arthritis in the distal parts of the legs and the tail. Abscesses[27] and abortions[28] may occur. Genetic factors seem to be essential for the susceptibility to the development of disease and perhaps for susceptibility to infection. Specifically, C57BL/6 mice seem to be highly susceptible, and the agent is difficult to isolate from nonsusceptible strains kept in the same unit as an infected susceptible strain.[26]

In guinea pigs, the agent causes local abscesses, which do not spread.[29] Although it is commonly mentioned in older literature that a biting pet rat died soon after the incident, this is unlikely to be related to the infection as the apathogenic nature of *S. moniliformis* in rats seems to be the basis for its successful spread.[25]

Table 14.2 Biochemical Characteristics of *Streptobacillus moniliformis*

D-Glucose	+	D-Xylose	–
Lactose	–	Citrate	–
Maltose	+	Indole	–
D-Mannitol	–	Nitrate reduction	–
Sucrose	–		

14.3.2.2 Characteristics of the agent

Cells of *S. moniliformis* are highly pleomorphic. Most cells are less than 1 µm wide and less than 5 µm long, but they may be up to 150 µm long. Branching is not observed. The colonies are 1 to 2 mm, round, grayish, smooth, and glistening. Also, fried-egg-type colonies may be observed.

Isolation media should be highly enriched (e.g., 20% serum should be added). Both agar and broth should be included in screening procedures. The blood or chocolate agars described in Chapter 3 may be applied; agars containing polyanetholesulfonate seem to be inhibitory.[30] The media are incubated microaerophilically (8% CO_2) for 1 to 6 days. Agars should be inspected daily for growth; broths should be inspected daily for so-called puffballs.

These bacteria are extremely difficult to isolate from healthy animals. The nose, trachea, and genitals are the most appropriate sites in healthy animals; joint fluid and blood should be included if disease is observed.

Streptobacillus moniliformis is the only important catalase-negative, Gram-negative, facultatively anaerobic rod in rodent bacteriology, and the micro-morphology also should be helpful in the identification process. It is oxidase negative and does not grow on MacConkey agar. Furthermore, the profile given in Table 14.2 may be used.

Enzyme-linked immunosorbent assay (ELISA) to measure *S. moniliformis* antibodies in mice and rats may be applied.[31] Different *S. moniliformis* strains originating from cases of rat bite fever in humans and from various rodent species show considerable serological relationship, but genetic differences in seroresponses also exist between different rat strains, with WKY and SHR high responders and BN and F344 low responders.[31] PCR and sequencing are also highly efficient as diagnostic tools.[32]

14.4 Candidate phylum TM7

No representatives of candidate phylum TM7 have to date been cultivated in the laboratory, and its existence is thus only known through first environmental 16S rRNA gene sequences and by sequencing of partial full genomes isolated using microfluidics-assisted single-cell genetics.[33,34] It

is a rod-shaped, filamentous and putatively Gram-positive bacterium.[33] It is a common member of the human oral microbiome and can also be detected in human fecal samples.[20,35] In mice, it is frequently detected in cecal and fecal samples, although seldom constituting more than 0.5% of the reads in high-throughput 16S rRNA gene amplicon sequencing-based studies.[20] Recently, it has been found that diet strongly influences the intestinal abundance of TM7 in nonobese diabetic (NOD) mice.[36] Pups born from pregnant NOD mice assigned to either a gluten-free or a gluten-containing diet were after birth cohoused with their mothers until weaning, after which the fecal gut microbiota of both mothers and pups were determined.[36] The mothers fed a gluten-free diet and their pups all had a much higher abundance of TM7 (6.062% vs. 0.012%) compared to NOD mice raised on a traditional chow diet.[36] Interestingly, the pups whose mothers had been fed the gluten-free diet later in life had significantly lower type 1 diabetes incidence compared to the chow diet group,[36] but whether TM7 actually plays a role in protection against type 1 diabetes in NOD mice remains to be elucidated.

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Appendix A: Producers of reagents for laboratory animal bacteriology

454 Life Sciences: http://www.454.com
Agilent Technologies: http://www.home.agilent.com
Anaerobe Systems: http://www.anaerobesystems.com
Applied Maths: http://www.applied-maths.com
Becton, Dickinson: http://www.bd.com
Biokar Diagnostics: http://www.biokar-diagnostics.com
Bioline: http://www.bioline.com
bioMérieux SA: http://www.biomerieux-diagnostics.com
Charles River Laboratories: http://www.criver.com
DAKO Corporation: http://www.dako.com
Difco. *See* Becton, Dickinson
Eurofins Genomics: http://www.eurofinsgenomics.eu
Fermentas. *See* Thermo Scientific
Gen-Probe Incorporated (Hologic): http://www.gen-probe.com
Gibco. *See* Life Technologies
Hardy Diagnostics: http://www.hardydiagnostics.com
Hologic. *See* Gen-Probe Incorporated
Illumina: http://www.illumina.com
Invitrogen. *See* Life Technologies

Lee Laboratories. *See* Becton, Dickinson
Life Technologies: http://www.lifetechnologies.com
Merck: http://www.emdgroup.com
Meridian Bioscience: http://www.meridianbioscience.com
Millipore: http://www.merckmillipore.com
Neogen: http://www.neogen.com
New Brunswick Scientific: http://newbrunswick.eppendorf.com
Oxoid: http://www.oxoid.com
Pacific Biosciences: http://www.pacificbiosciences.com
PerkinElmer: http://www.perkinelmer.com
Qiagen: http://www.qiagen.com
Roche Diagnostics: http://www.roche.com
Rosco Diagnostica: http://www.rosco.dk
Sigma-Aldrich: http://www.sigma-aldrich.com
Strategene. *See* Agilent Technologies
Thermo Scientific: http://www.thermoscientificbio.com

Appendix B: Biosafety levels for microbiological laboratories

Bacteria posing a risk to laboratory staff should be handled with certain precautions. The US Centers for Disease Control and Prevention (CDC)[1] has set up four biosafety levels for work with microorganisms. These criteria should be consulted for work performed outside US borders as well. The European Union directive is slightly different on some points, but basically the principles for protection are the same.[2] The information in this appendix is, in principle, a short review of these recommendations,[1,2] but the requirements should be known in detail before any microbiological work is initiated. A short visual introduction to these levels may be obtained by watching the introduction to the movie *Outbreak* (Warner Bros.), available on YouTube.[3]

It should be noted that different procedures for the same agent may require different biosafety levels. In general, procedures are divided into three different types:

- The use or manipulation of known or potentially infectious tissues, body fluids, and cultures
- Housing and handling infected animals
- Work involving production volumes or concentrations of cultures

Biosafety level 1 (Table B.1) is sufficient for work involving well-characterized agents not known to cause disease in healthy adult humans and of minimal potential hazard to laboratory personnel and the environment. The laboratory is not necessarily separated from the general traffic

Table B.1 Standard and Special Safety Practices, Equipment, and Facilities for Work with Agents Assigned to Biosafety Level 1

A. **Standard Microbiological Practices**
   1. Limited access to laboratory.
   2. Hand washing after handling viable materials and animals, after removing gloves, and before leaving the laboratory.
   3. No eating, drinking, smoking, handling contact lenses, or applying cosmetics in the laboratory. Persons who wear contact lenses in laboratories are to wear goggles or a face shield. Food to be stored outside the work area in cabinets or refrigerators designated and used for this purpose only.
   4. No mouth pipetting.
   5. Avoid creating splashes or aerosols.
   6. Daily decontamination of work and after any spill of viable material.
   7. Decontamination of all cultures, stocks, and other regulated wastes before disposal by an approved decontamination method, such as autoclaving. Materials for decontamination outside of the immediate laboratory are to be placed in a durable leakproof container and closed for transport from the laboratory. Materials for decontamination off site from the laboratory to be packaged in accordance with applicable local, state, and federal regulations before removal from the facility.
   8. Insect and rodent control program in effect.

B. **Special Practices:** None

C. **Safety Equipment (Primary Barriers)**
   1. Laboratory coats, gowns, or uniforms to prevent contamination or soiling of street clothes.
   2. Gloves, if the skin on the hands is broken or if a rash exists.
   3. Protective eyewear for anticipated splashes of microorganisms or other hazardous materials to the face.

D. **Laboratory Facilities (Secondary Barriers)**
   1. A sink for hand washing in each laboratory.
   2. Easy cleaning design.
   3. Bench tops impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.
   4. Sturdy laboratory furniture. Spaces between benches and cabinets and equipment accessible for cleaning.
   5. Fly screens on all windows to open (if any).

Source: US Department of Health and Human Services, Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical Laboratories*. 5th edition. HHS Publication No. (CDC) 21-1112. Atlanta, GA: CDC, 2009. The original paper is found on http://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf

patterns in the building. Work is generally conducted on open bench tops using standard microbiological practices. Special containment equipment or facility design is not required or generally used. Laboratory personnel have specific training in procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or a related science.[1] Biosafety level 1 covers the majority of the procedures and the agents discussed in this book.

Biosafety level 2 (Table B.2) standards are similar to level 1 and suitable for work involving agents of moderate potential hazard to personnel and the environment. Biosafety level 2 criteria differ from level 1 as follows:

- Laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists.
- Access to the laboratory is limited when work is being conducted.
- Extreme precautions are taken with contaminated sharp items.
- Certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets or other physical containment equipment.[2]

Biosafety level 2 is applied for agents such as *Clostridium difficile* and *Salmonella* spp.

Table B.2 Standard and Special Safety Practices, Equipment, and Facilities for Work with Agents Assigned to Biosafety Level 2

A. **Standard Microbiological Practices:** As for Biosafety Level 1

B. **Special Practices**

1. No persons who are at increased risk of acquiring infection or for whom infection may be unusually hazardous.
2. Only persons advised of the potential hazard and who meet specific entry requirements.
3. Special provisions for entry and a hazard warning sign (the universal biohazard symbol, name of the infectious agent, name and telephone number of the responsible person[s], and the special requirement[s] for entering the laboratory) on the access door.
4. Staff immunizations or tests for the agents handled or potentially present in the laboratory.
5. Possibly serum samples from laboratory and other at-risk personnel.
6. A biosafety manual.
7. Appropriate staff training, including annual updates on potential hazards, necessary precautions, and exposure evaluation procedures.
8. Precaution with any contaminated sharp items, including needles and syringes, slides, pipettes, capillary tubes, and scalpels. Plasticware instead of glassware whenever possible.

continued

Table B.2 (continued) Standard and Special Safety Practices, Equipment, and Facilities for Work with Agents Assigned to Biosafety Level 2

9. Cultures, tissues, or specimens of body fluids to be placed in a container that prevents leakage during collection, handling, processing, storage, transport, or shipping.
10. Laboratory equipment and work surfaces to be decontaminated with an appropriate disinfectant on a routine basis, after work with infectious materials, and especially after overt spills, splashes, or other contamination by infectious materials. Contaminated equipment to be decontaminated before removal from the facility.
11. Major spills and accidents to be reported immediately to the laboratory director.
12. No animals other than those involved in the work.

C. **Safety Equipment (Primary Barriers)**
   1. Properly maintained biological safety cabinets (BSCs), preferably Class II, or other appropriate personal protective equipment or physical containment devices to be used whenever
      a. Procedures with a potential for creating infectious aerosols or splashes are conducted.
      b. High concentrations or large volumes of infectious agents are used.
   2. Face protection to be used for anticipated splashes or sprays of infectious or other hazardous materials to the face, when the microorganisms must be manipulated outside BSCs.
   3. Protective laboratory coats, gowns, smocks, or uniforms designated for lab use to be worn while in laboratory and removed and left in the laboratory before leaving.
   4. Gloves to be worn when handling infected animals and when hands may contact infectious materials, contaminated surfaces, or equipment.

D. **Laboratory Facilities (Secondary Barriers):** As for biosafety level 1 with the following addition:
   A method for decontamination of infectious or regulated laboratory wastes and an eyewash facility to be available.

Source: US Department of Health and Human Services, Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical Laboratories*. 5th edition. HHS Publication No. (CDC) 21-1112. Atlanta, GA: CDC, 2009. The original paper is found on http://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf

Biosafety level 3 (Table B.3) is applicable to clinical, diagnostic, teaching, research, or production facilities where work is done with indigenous or exotic agents that may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents and are supervised by competent scientists who are experienced in working with these agents. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets or other physical containment

Table B.3 Standard and Special Safety Practices, Equipment, and Facilities for Work with Agents Assigned to Biosafety Level 3

A. **Standard Microbiological Practices:** As for Biosafety Level 1.

B. **Special Practices:** As for Biosafety Level 2 with the following additions:

1. All personnel to demonstrate proficiency in standard microbiological practices and techniques and in the practices and operations specific to the laboratory facility.
2. All manipulations involving infectious materials to be conducted in biological safety cabinets (BSCs) or other physical containment devices within the containment module. No work in open vessels to be conducted on the open bench.
3. Spills of infectious materials to be decontaminated, contained, and cleaned up by appropriate professional staff.

C. **Safety Equipment (Primary Barriers)**

1. Properly maintained BSCs (class II or III) to be used for all manipulation of infectious materials.
2. Outside a BSC, appropriate combinations of personal protective equipment in combination with physical containment devices to be used for manipulations of any material (live or dead) that may possibly be a source of infectious aerosols and for sampling and necropsying infected animals or embryonated eggs.
3. Face protection to be worn for manipulation of infectious materials outside BSCs.
4. Respiratory protection to be worn when aerosols cannot be safely contained (i.e., outside BSCs) and in rooms containing infected animals.
5. Protective laboratory clothing to be worn in, and not worn outside, the laboratory.
6. Gloves to be worn when handling infected animals and when hands may contact infectious materials and contaminated surfaces or equipment.
7. Reusable laboratory clothing to be decontaminated before being laundered. Disposable gloves to be discarded when contaminated and never washed for reuse.

D. **Laboratory Facilities (Secondary Barriers):** As for biosafety level 2 with the following additions:

1. The laboratory to be separated from areas that are open to unrestricted traffic flow within the building.
2. Passage into the laboratory through two sets of self-closing doors and possibly a clothes-changing room (shower optional).
3. A ducted exhaust air ventilation system based on a directional airfoil that draws air from "clean" areas into the laboratory toward "contaminated" areas, no recirculation to any other area, and discharge to the outside with filtration and other treatment optional. The outside exhaust to be dispersed away from occupied areas and air intakes.

continued

Table B.3 (continued) Standard and Special Safety Practices, Equipment, and Facilities for Work with Agents Assigned to Biosafety Level 3

4. An eyewash facility.
5. The high-efficiency particulate air (HEPA)-filtered exhaust air from BSCs to be discharged directly to the outside or through the building exhaust system.
6. Equipment that may produce aerosols to be contained in devices that exhaust air through HEPA filters before discharge into the laboratory.
7. Vacuum lines to be protected with liquid disinfectant traps and HEPA filters or their equivalent.

Source: US Department of Health and Human Services, Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical Laboratories*. 5th edition. HHS Publication No. (CDC) 21-1112. Atlanta, GA: CDC, 2009. The original paper is found on http://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf

devices or by personnel wearing appropriate personal protective clothing and equipment. The laboratory has special engineering and design features.

Biosafety level 3 and animal biosafety level 3 practices, containment equipment, and facilities are recommended for all manipulations of cultures and for experimental animal studies involving *Francisella tularensis* and for the propagation and manipulation of cultures of *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

Biosafety level 4 is required for work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease. Agents with a close or identical antigenic relationship to biosafety level 4 agents are handled at this level until sufficient data are obtained either to confirm continued work at this level or to work with them at a lower level. Biosafety level 4 is not mandatory for any agent listed in this book and therefore is not described in further detail here.

References

1. US Department of Health and Human Services, Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical Laboratories*. 5th edition. HHS Publication No. (CDC) 21-1112. Atlanta, GA: CDC, 2009.
2. European Union. *Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the Protection of Workers from Risks Related to Exposure to Biological Agents at Work (Seventh Individual Directive within the Meaning of Article 16(1) of Directive 89/391/EEC).* Brussels: European Union, 2000.
3. Petersen W, director. *Outbreak* introduction. 1995. https://www.youtube.com/watch?v=-1di7g4Hm1s.

Appendix C: Media buffers and reagents commonly applied in laboratory animal bacteriology

Table C.1 Example of a Recipe for 5% Blood Agar[a]

Sterile, deionized water	1000 ml
Magnesium sulfate, $7H_2O$	0.1 g
Manganese chloride, $7H_2O$	0.0067 g
Disodium hydrogen phosphate, $12H_2O$	0.0067 g
Casein hydrolysate	5.0 g
Yeast extract	3.0 g
Potassium chloride	6.67 g
Sebacic acid	0.01 g
Agar	10.0 g
Peptone	5.0 g
Defibrinized horse blood	50 ml
Cysteine HCL	0.05 g
Sodium pyruvate	2.0 g

Note: pH is stabilized at 7.4.

[a] An agar base, which only has to be supplied with blood, is available from most producers of bacteriological media and may be used as an alternative.

Table C.2 Semisolid Medium for Testing Motility of Bacteria

Gelatin	80 g
Distilled water	1000 ml
Peptone	10 g
Beef extract	3 g
Sodium chloride	5 g
Agar	4 g

Table C.3 Kovac's Reagent for Testing Cytochrome Oxidase Activity

Tetramethyl-*p*-phenylene-diamine	1 g
Ascorbic acid	1 g
Distilled water	1000 ml

Source: Kovacs N. Identification of *Pseudomonas-pyocyanea* by the oxidase reaction. *Nature* 1956; 178:703.

Table C.4 Broth for Testing Carbohydrate Fermentation

Beef extract	5.0 g
Bromothymol blue	0.024 g
Carbohydrate	5.0 g
Disodium hydrogen phosphate	2.0 g
Peptone	10.0 g
Sodium chloride	3.0 g

Note: After inoculation, the broth should be incubated at 37°C for up to 1 week and inspected daily. A change in color from blue to yellow is interpreted as positive.

Table C.5 Simple Reagent Usable for Creating an Anaerobic Environment in a Jar or Sealed Plastic Bag

Pyrogallol	50 g
Potassium carbonate	50 g
Terra silica	250 g

Note: The compounds are efficiently mixed together. Can be maintained in packages of approximately 2 g in an exicator for about 1 month.

Table C.6 Example of a Recipe for Chocolate Agar[a]

Sterile, deionized water	1000 ml
Peptone	15.0 g
Yeast extract	1.0 g
Sodium chloride	5.0 g
Citric acid	0.15 g
Dipotassium hydrogen phosphate	0.0067 g
Starch	2.0 g
Agar	10.0 g
Nicotine amide dinucleotide (NAD)	0.0004 g
Autolyzed liver	20.0 ml
Glucose	1.5 g
Defibrinized horse blood	70 ml[b]

Source: Leifson E. Types of bacteria on blood and chocolate agar and the immediate cause of these types. *J Bacteriol* 1932; 24:473–487.

Note: pH is stabilized at 7.2.

[a] If the agar is to be used for anaerobic cultivation, 0.001 g of vitamin K and 0.55 g of cysteine HCL per 1000 ml are added to support growth of *Bacteroides* spp.

[b] Briefly heat the blood shortly before use.

Table C.7 Recipe for Mueller Hinton Agar to Be Used for Agar Diffusion Inhibition Assays[a]

Water	1000 ml
Peptone 5 (acid digest of casein)	21.5 g
Beef infusion	2.0 g
Agar	13.0 g

Source: Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; 45:493–496.

Note: The mixture is boiled to dissolve and then autoclaved. After cooling to 50°C, 5% v/v horse or sheep blood may be added if necessary for growth. The pH should be adjusted to 7.1 to 7.5.

[a] The agar base is commercially available from Becton Dickinson, Oxoid, Neogen, and others.

Table C.8 Medium for Observation of Spores in *Bacillus* spp.[a]

Dehydrated nutrient broth	4 g
Yeast extract	8 g
MnCl, 4H2O	0.01 g
Agar	2 g
Distilled water until	1000 ml

[a] The medium is incubated at temperatures according to the temperature optimum for the species in question and examined for sporulation twice per day.

Table C.9 Triple-Sugar Iron Slant for Differentiating *Erysipelothrix rhusiopathiae* from Other Gram-Positive Rods

Meat extract	3 g
Yeast extract	3 g
Peptone	20 g
Glucose	1 g
Lactose	1 g
Sucrose	10 g
$FeSO_4 \cdot 7H_2O$	0.2 g
NaCl	5 g
$Na_2S_2O_3 \cdot 5H_2O$	0.3 g
Agar	20 g
Water	1000 ml
Phenol red, 0.2% in aqueous solution	12 ml

Source: Cox NA, Williams JE. Simplified biochemical system to screen *Salmonella* isolates from poultry for serotyping. *Poult Sci* 1976; 55:1968–1971.

Note: After mixing and sterilization, the mixture is dispensed into tubes to form slopes with deep butts about 3 cm long.

Table C.10 Recipe for Baird Parkers Agar[a]

Water	1000 ml
Peptone 140 (pancreatic digest of casein)	10.0 g
Peptone 190 (pancreatic digest of gelatin)	5.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lithium chloride	5.0 g
Agar	15.0 g

Source: Baird-Parker AC. An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J Appl Bacteriol* 1962; 25:12–19.

Note: The mixture is boiled to dissolve and autoclaved. After cooling to 50°C, 0.1% w/v potassium tellurite and 50 ml egg yolk emulsion are added. pH should be adjusted to 7.0 to 7.2.

[a] The medium is incubated aerobically at 37°C. Lithium chloride prevents growth of Gram-negative bacteria, and tellurite prevents growth of coagulase-negative staphylococci. *Staphylococcus aureus* and other coagulase-positive staphylococci form black colonies because of reduction of tellurite, surrounded by a zone, which at first appears clear because of lipo- and proteolysis, but later becomes unclear because of lecithinase and lipase activity. Identification is not 100% specific and should be confirmed by other means.

Table C.11 Mannitol Salt Agar, a Valuable Medium for the Isolation of Staphylococci[a]

Water	1000 ml
Peptone	10.0 g
Beef extract	1.0 g
NaCl	75.0 g
Mannitol	10.0 g
Phenol red	0.025 g
Agar	15.0 g

Source: Chapman GH. The significance of sodium chloride in studies of *Staphylococci*. *J Bacteriol* 1945; 50:201–203.

Note: pH should be adjusted to 7.2 to 7.4.

The agar is commercially available from Becton Dickinson, Neogen, and others.

[a] *Staphylococcus aureus* ferments mannitol and produces a golden pigment.

Table C.12 Phenol Red Acriflavin Lithium Chloride Columbia Agar with Mannitol (PALCAM Agar) to Be Used for Cultivating *Listeria* spp.[a]

Water	1000 ml
Peptone	23.0 g
Starch	1.0 g
NaCl	5.0 g
Agar	25.0 g
Phenol red	0.08 g
Acriflavin	0.005 g
Lithium chloride	15.0 g
Esculin	0.75 g
Ferric ammonium citrate	0.5 g
D-Mannitol	10.0 g
Polymyxin B	81.654 IU
Ceftazidim	0.02 g

Source: Van Netten P, Perales I, Van De Moosdijk A, Curtis GDW, Mossel DAA. Liquid and solid selective differential media for the detection and enumeration of *Listeria monocytogenes* and other *Listeria* spp. *Int J Food Microbiol* 1989; 8:299–316.

Note: pH is adjusted to 7.2.

[a] After approximately 48 h, *L. monocytogenes* will form black colonies, which after some time turn green, occasionally with a metallic appearance.

Table C.13 Cycloserine-Cefoxitin Egg Yolk (CCEY) Agar for Selective Cultivation of *Clostridium difficile*[a]

Water	1000 ml
Peptone	40.0 g
NaCl	2.2 g
Disodium hydrogen phosphate, $2H_2O$	6.3 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulfate, $7H_2O$	0.2 g
Neutral red	0.03 g
D-Fructose	6.0 g
Cycloserine	0.5 g
Cefoxitin	0.016 g
Pasteurized egg yolk	25 ml
Agar	20.0 g

Source: George WL, Sutter VL, Citron D, Finegold SM. Selective and differential medium for isolation of *Clostridium-difficile*. *J Clin Microbiol* 1979; 9:214–219.

Note: pH is adjusted to 7.0.

[a] After 48 h of anaerobic incubation, colonies of *C. difficile* are 5 to 8 mm, are spreading, and have a rough surface, which under ultraviolet light reflects a yellow-green fluorescence. Furthermore, the colonies smell a little like horse stables, which, however, is not fully specific for *C. difficile.*

Table C.14 Egg Yolk Agar (EYA) for Initial Examination of *Clostridium* spp.[a]

Water	1000 ml
Peptone	40.0 g
NaCl	2.0 g
Disodium hydrogen phosphate	5.0 g
$MgSO_4$, $7H_2O$	0.02 g
Glucose	2.0 g
Pasteurized egg yolk	25 ml
Agar	25.0 g

Source: McClung LS, Toabe R. The egg yolk plate reaction for the presumptive diagnosis of *Clostridium-sporogenes* and certain species of the gangrene and botulinum groups. *J Bacteriol* 1947; 53:139–147.

Note: pH is adjusted to 7.3.

The agar is commercially available from Anaerobe Systems, Becton Dickinson, and others.

[a] After 72 to 96 h of incubation, the following will be observed: lecithinase activity; lipase activity; halo around the colony; oily, shining surface of the colony.

Table C.15 Recipe for MacConkey Agar[a]

Sterile, deionized water	1000 ml
Peptone	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Ox bile	106 ml
Neutral red	0.075 g
Agar	9.0 g

Source: MacConkey AT. Note on a new medium for the growth and differentiation of the bacillus *Coli communis* and the bacillus *Typhi abdominalis*. *Lancet* 1900; 2:20.

Note: pH is stabilized at 7.2.

[a] The agar is commercially available from Oxoid and others.

Table C.16 Recipe for Selenite Broth[a]

Sterile, deionized water	1000 ml
Peptone	5.0 g
Lactose	4.0 g
Sodium hydrogen selenite	6.0 g
Disodium hydrogen phosphate, $12H_2O$	10.0 g

Source: Leifson E. New selenite enrichment media for the isolation of typhoid and paratyphoid (*Salmonella*) bacilli. *Am J Hyg* 1936; 24:423–432.

Note: pH is stabilized at 7.0.

[a] Samples are inoculated directly into 7 ml of the broth and incubated at 37°C aerobically for 12 to 24 h, after which an indicative agar is streaked from the broth. The broth is commercially available from Oxoid, Becton Dickinson, and others.

Table C.17 Recipe for Brilliant-Green Phenol-Red Lactose-Sucrose (BPLS) Agar[a]

Sterile, deionized water	1000 ml
Peptone 140 (pancreatic digest of casein)	7.0 g
Peptone 100 (pancreatic digest of animal tissue)	7.0 g
Yeast extract	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Sodium chloride	5.0 g
Phenol red	0.04 g
Brilliant green	0.004 g
Agar	14.0 g

Source: Kristensen M, Lester V, Jurgens A. On the use of trypsinized casein, brom-thymol-blue, brom-cresol-purple, phenol-red and brilliant-green for bacteriological nutrient media. *Br J Exp Pathol* 1925; 6:291–299.

Note: Should only be boiled to dissolve the components. pH is stabilized at 6.9.

[a] Gram-positive and most Gram-negative bacteria, except for *Salmonella*, are inhibited. *Salmonella* grows as red colonies; lactose/sucrose fermenters, such as *Proteus*, which will occasionally grow on this agar, occur as yellow colonies. Also, *Pseudomonas* occasionally grows with red colonies but may easily be differentiated if a colony is subjected to a test for cytochrome oxidase. The agar is commercially available from Becton Dickinson or Merck, for example.

Table C.18 King's Agars A and B for Visualizing Pigment Production by *Pseudomonas* spp.[a]

King's Agar A (Slants)[b]	
Sterile, deionized water	1000 ml
Peptone	20.0 g
Glycerol	8.7 g
Potassium sulfate	10.0 g
Magnesium sulfate, $6H_2O$	3.0 g
Agar	15.0 g
King's Agar B (Slants)[c]	
Sterile, deionized water	1000 ml
Peptone	20.0 g
Glycerol	8.7 g
Dipotassium phosphate	1.5 g
Magnesium sulfate, $7H_2O$	1.5 g
Agar	15.0 g

Source: King EO, Ward MK, Raney DE. Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* 1954; 44:301–307.

Note: The agar is commercially available from Becton Dickinson, Neogen, Biokar, and others.

[a] If King's agar A turns blue, the inoculated isolate is a pyocyanin-producing *P. aeruginosa*. King's agar B is inspected with a Woods lamp. If fluorescence is observed, the inoculated isolate produces fluorescein, which is typical for *P. aeruginosa*, *P. fluorescens,* and *P. putida.*

[b] pH is stabilized at 7.2.

[c] pH is stabilized at 7.4.

Table C.19 Trimethoprim, Vancomycin, Polymyxin B (TVP) Agar for the Isolation of *Campylobacter* and *Helicobacter* spp.

Sterile, deionized water	1000 ml
Magnesium sulfate, $7H_2O$	0.1 g
Manganese chloride, $4H_2O$	0.007 g
Disodium hydrogen phosphate, $12H_2O$	8.0 g
Casein hydrolysate	5.0 g
Yeast extract	3.0 g
Peptone	5.0 g
Potassium chloride	6.7 g
Sebacic acid	0.01 g
Agar	10.0 g
Cysteine HCl	0.05 g
Defibrinized horse blood	50 ml
Sodium pyruvate	2.0 g
Trimethoprim	0.005 g
Vancomycin	0.01 g
Polymyxin B	2500 IU

Sources: Hanninen ML, Jalava K, Saari S, Happonen J, Westermarck E. Culture of *Gastrospirillum* from gastric biopsies of dogs. *Eur J Clin Microbiol Infect Dis* 1995; 14:145–146; Røder, BL, *Handbook of Culture Media*, SSI, Media Department, ISBN-10: 87-89148-40-1, 1993. With permission.

Note: pH is stabilized at 7.2.

Table C.20 Recipe for Löwenstein–Jensen Slants

Sterile, deionized water	612 ml
l-Asparagine	3.6 g
Potassium dihydrogen phosphate	2.4 g
Magnesium citrate	0.6 g
Glycerin	12 ml
Malachite green	0.4 g
Magnesium sulfate, $7H_2O$	0.24 g
Homogenized egg	1000 ml

Source: Lowenstein E. Der kulturelle Nacheweis von Tuberkelbakterien in Milch auf Malachitgrun Einahrboden. *Ann Inst Pasteur* 1933; 50:161.

Note: pH is stabilized at 6.8 to 7.2. The medium is filled into tubes as slants.

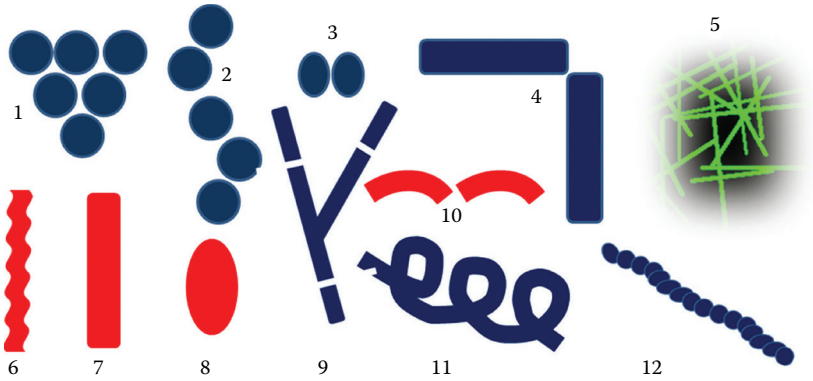


Figure 3.2

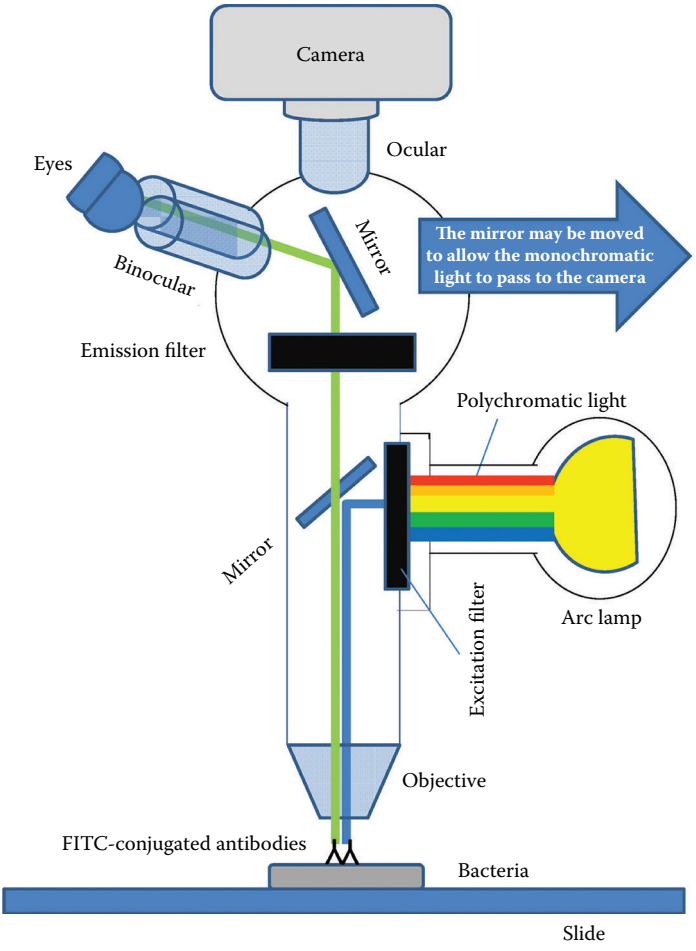


Figure 4.3

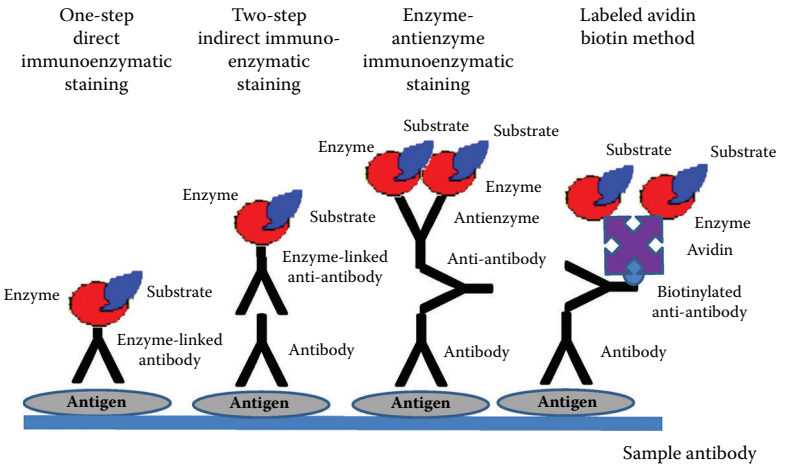


Figure 4.4

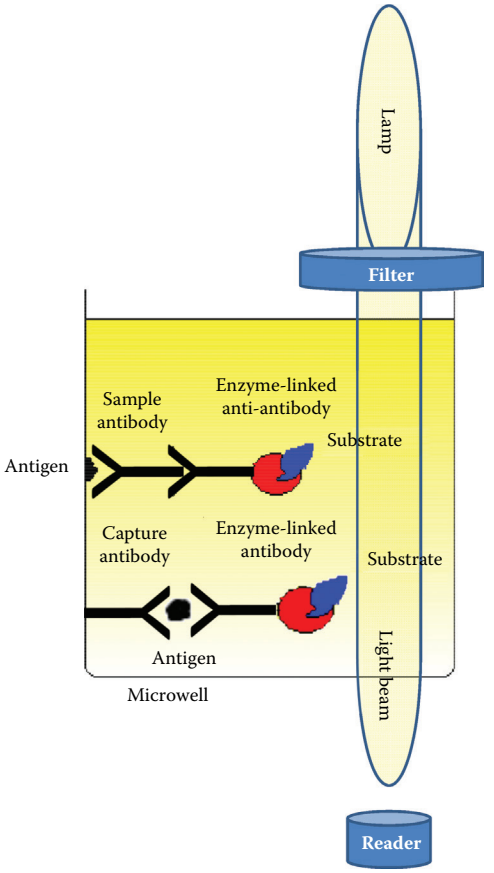


Figure 4.5

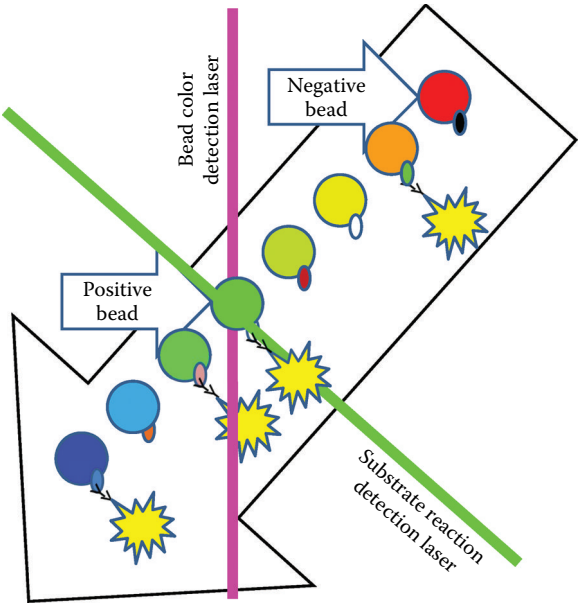


Figure 4.8

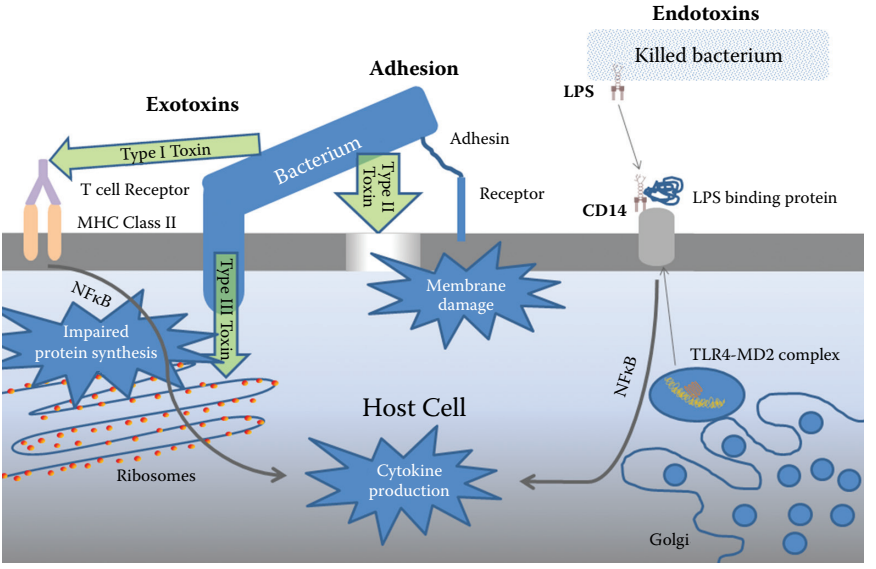


Figure 6.1

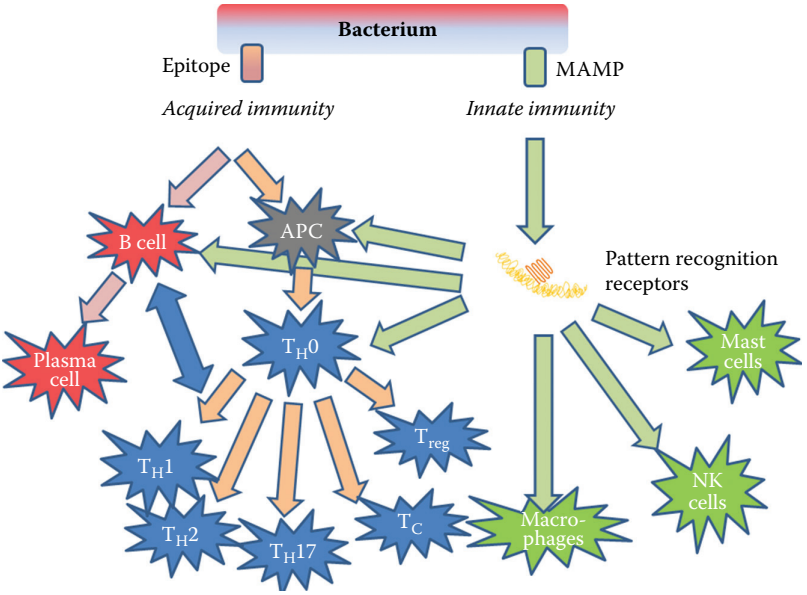


Figure 6.2

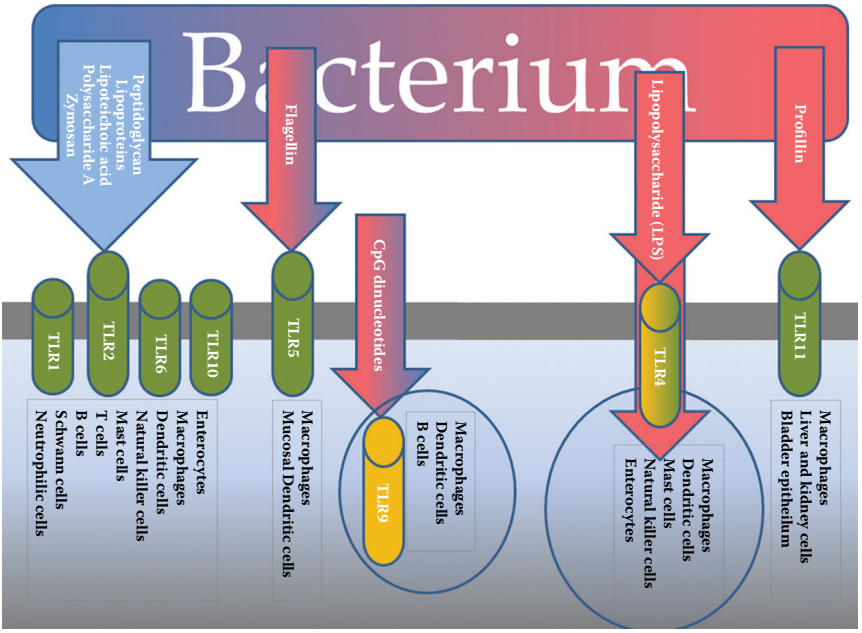


Figure 6.3

The **Handbook of Laboratory Animal Bacteriology, Second Edition** provides comprehensive information on all bacterial phylae found in laboratory rodents and rabbits to assist managers, veterinary pathologists, and laboratory animal veterinarians in the management of these organisms. The book starts by examining the general aspects of bacteriology and how to sample and identify bacteria in animals. It then describes the most relevant species within each phylum and discusses the impact they may have on research. Emphasizing those bacteria known to interfere with research protocols, the book offers methods for isolation and differentiation among related bacteria. It discusses where to purchase reagents for rodent bacteriology and outlines standards for safety in a bacteriological laboratory.

Highlights of the second edition:

- Focuses on modern sequencing techniques based on molecular identification
- Reorganizes content according to modern systematics based on new identification methods
- Presents new chapters on mechanisms behind bacterial impact on animal models and on the systematic classification of bacteria
- Provides information on a range of bacteria interfering with animal models for human disease, not only for those bacteria which cause disease in laboratory animal colonies
- Includes new figures in color and with enhanced resolution

The book is essential reading for those interested in the management of organisms known to interfere with the colony health of rabbits and rodents used in research protocols—including facility managers, clinical veterinarians, veterinary pathologists, and researchers.

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