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PCR and RT-PCR in the Diagnosis of Laboratory Animal Infections and in Health Monitoring

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Molecular diagnostics (PCR and RT-PCR) have become commonplace in laboratory animal research and diagnostics, augmenting or replacing serological and microbiologic methods. This overview will discuss the uses of molecular diagnostics in the diagnosis of pathogenic infections of laboratory animals and in monitoring the microbial status of laboratory animals and their environment. The article will focus primarily on laboratory rodents, although PCR can be used on samples from any laboratory animal species.

Abbreviations: IVC, individually ventilated cage; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; MAP, mouse antibody production; MHV, mouse hepatitis virus; MNV, murine norovirus; MPV, mouse parvovirus

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The use of molecular methods (PCR and RT-PCR) to diagnose bacterial, viral, fungal and parasitic infections in laboratory animals has become common, frequently replacing or augmenting serological, microscopic and culture-based methods.[24] Because the majority of laboratory animals are rodents, this review will focus on the detection of infectious agents in rodents and their environment, but PCR and RT-PCR testing of many other commonly used laboratory species such as rabbits, dogs, cats, ferrets, pigs, fish, frogs, and nonhuman primates are commercially available. Recommendations on which infectious agents to test for and exclude from mouse and rat colonies, and the frequency with which the testing should be performed, have been developed by FELASA and AALAS.[44,117] PCR is used to monitor for over 60 rodent infectious agents (Figure 1).[18,44,71] FELASA health monitoring guidelines for hamsters, guinea pigs, rabbits, and nonhuman primates have also been published.[4,44]

PCR or RT-PCR can overcome many of the limitations of other methods of pathogen detection (Figure 2) and therefore represent a refinement of health monitoring in the vivarium. Briefly, PCR-based diagnostic methods amplify the genome of viruses present in, or released from, infected cells or the DNA of bacteria, fungi, or parasites.[24,53] Three advantages of PCR are its speed, sensitivity, and specificity; unlike serologic or microbiologic techniques, molecular diagnostics can amplify a specific region of a gene from a few copies to trillions in a few hours. However, the high sensitivity of PCR can lead to false positives if samples or reagents are contaminated with nucleic acids from the infectious agent (positive controls) or products from recent PCR assays.[80] Because RNA is prone to degradation by RNases, RT-PCR can be prone to false negatives if care is not used to prevent RNA degradation during sample collection and RNA extraction. PCR is versatile and can be used on tissue samples or excreta from any species of animal or from samples taken from the laboratory animal environment. Collecting samples for PCR testing requires knowledge of which tissues become infected with the agent of interest and where the pathogen is shed. The DNA primers selected for PCR determine its specificity.[24,53] Primers are designed based on available sequence data and can either amplify a single species of an agent (for example, *Mycoplasma pulmonis*) or amplify several related agents (all *Mycoplasma* species). For rapidly evolving agents such as RNA viruses, a nucleotide change in the primer region can lead to a false-negative result in that the agent is present in the sample, but the targeted gene is not amplified.

This review will not discuss methods for nucleic acid extraction and amplification, or their limitations, as these methods have been published previously[53,106] and are provided with commercial nucleic acid extraction and amplification kits.

Viral infections

The clinical signs and/or histopathology produced by some viruses are specific to a single virus. For example, the characteristic dermal lesions of Ectromelia virus,[48] the chromodacryorrhea and sneezing caused by rat coronavirus,[10] the enterocyte syncytia in young mice caused by mouse hepatitis virus (MHV)[5] or the acidophilic intranuclear inclusions in the salivary glands cause by mouse and rat cytomegalovirus[15] can all be used to make a presumptive diagnosis. However, viruses that cause severe distinctive lesions essentially have been eliminated from laboratory rodent colonies, resulting in focus on viruses that cause little or no pathology or morbidity. Therefore, diagnosis of viral infections in rodents using serology has become the most common method of diagnosis.[24,55,129]

Serologic methods detect antibodies specific for the infectious agent in blood collected from an animal. Serology is an indirect method for detecting infection; therefore, knowing the tissue that harbors the infection is not necessary. Because species-specific secondary antibodies are used in most serology tests, they are limited to those species for which commercial sources of species-specific antibodies are available.

Virus-specific antibodies are first detected in immunocompetent mice 5 to 7 d after infection and peak levels of antibodies are detected 10 to 20 d after infection. Because antibodies may persist for months, a seropositive animal may not be acutely

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Infectious Agent	Agent Type	Monitored in Mice	Monitored in Rats
Boone cardiovirus	Virus		X
Bordetella bronchoseptica	Bacteria	X	X
Bordetella hinzii	Bacteria	X	
Bordetella pseudohinzii	Bacteria	X	
Campylobacter	Bacteria	X	X
Chilomastix	Parasite	X	X
Citrobacter rodentium	Bacteria	X	
Clostridium piliforme	Bacteria	X	X
Corynebacterium bovis	Bacteria	X	
Corynebacterium kutscheri	Bacteria	X	X
Cryptosporidium	Parasite	X	X
Dermatophytes	Fungi	X	X
Ectromelia virus	Virus	X	
Encephalitizoon cuniculi	Fungi		X
Entameoba muris	Parasite	X	X
Filobacterium rodentium	Bacteria	X	X
Fur mites (Myobia/Myocoptes/Radfordia)	Parasite	X	X
Giardia muris	Parasite	X	X
H-1 parvovirus	Virus		X
Hantaviruses (Hantaan/Seoul)	Virus	X	X
Helicobacter	Bacteria	X	X
K virus	Virus	X	
Klebsiella oxytoca	Bacteria	X	X
Klebsiella pneumoniae	Bacteria	X	X
Lactate dehydrogenase-elevating virus	Virus	X	
Lymphocytic choriomeningitis virus	Virus	X	
Minute virus of mice	Virus	X	
Mouse hepatitis virus	Virus	X	
Mouse kidney parvovirus	Virus	X	
Mouse parvovirus	Virus	X	
Murine polyomavirus	Virus	X	
Mouse thymic virus	Virus	X	
Murine adenoviruses 1 and 2	Virus	X	X
Murine chapparvovirus	Virus	X	
Murine cytomegalovirus	Virus	X	
Murine norovirus	Virus	X	
Murine rotavirus	Virus	X	
Mycoplasma pulmonis	Bacteria	X	X
Other mites (Demodex/Ornithonyssus)	Parasite	X	X
Pinworms (Aspiculuris/Syphacia)	Parasite	X	X
Pneumocystis murina	Fungi	X	X
Pneumonia virus of mice	Virus	X	X
Proteus mirabilis	Bacteria	X	X
Pseudomonas aeruginosa	Bacteria	X	X
Rat coronavirus	Virus		X
Rat minute virus	Virus		X
Rat parvovirus	Virus		X
Rat polyomavirus 2	Virus		X
Rat virus	Virus		X
Reovirus	Virus	X	X
Rodentibacter pneumotropicus and heylii	Bacteria	X	X
Salmonella	Bacteria	X	X
Sendai virus	Virus	X	X
Spironucleus muris	Parasite	X	X
Staphylococcus aureus	Bacteria	X	X
Staphylococcus xylosus	Bacteria	X	X
Streptobacillus moniliformis	Bacteria	X	X
Streptococcus, β hemolytic	Bacteria	X	X
Streptococcus pneumoniae	Bacteria	X	X
Theilovirus	Virus	X	X
Tritrichomonas muris	Parasite	X	X

Data derived from FELASA, Charles River and IDEXX recommended panels {references 18, 44 and 71}

Figure 1. Infectious agents recommended for monitoring in mice and rats.

infected and the infectious agent may have already been cleared. The presence of viral antibodies in a weanling mouse or rat may represent transfer of maternal antibodies from a previously infected dam, rather than infection of the weanling. Serologic testing is not appropriate for immunocompromised or neonatal mice that do not mount an adequate antibody response. While

	Pathogen types detected	Used with environmental samples	Pooled samples	Used in wide range of animal species	Time to diagnosis	Pathogen identification
PCR/RT-PCR	Viruses, Bacteria, Fungi, Parasites	Yes	Yes	Yes	Hours	Pathogen-specific primers and sequencing
Histopathology	Viruses, Bacteria, Fungi, Parasites	No	No	Yes	Days	Visualization of lesions in stained tissues
Growth in/on culture media	Bacteria and Fungi	Yes	Yes	Yes	Days to weeks	Growth conditions, stains, biochemical testing of isolates
Growth in cultured cells	Primarily Viruses	No	No	Restricted by culture cell type availability	Days to weeks	General identification based on cell type and cytopathic effects observed
Serology	Primarily Viruses and Selected Bacteria and Parasites	No	Yes	Restricted by species specific secondary antibody availability	Hours (if infection is at least 5 days old)	Pathogen-specific antibodies
Electron microscopy	Primarily Viruses	No	No	Yes	Days	Visualization of pathogen
Light microscopy	Parasites	No	Yes	Yes	Hours	Visualization of pathogen

Figure 2. Laboratory animal infectious agent detection methods.

serological responses can be quantified, a stronger antibody response does not correlate with the presence of higher viral loads in the animal. Soiled-bedding sentinel serology is used in many laboratory rodent facilities for health monitoring, as sera from a single sentinel can be used to determine whether an infection occurred in any of the cages of experimental mice or rats from which soiled bedding was collected. A limitation of soiled bedding sentinels is that viruses that are not transmitted via the fecal-oral route, such as Sendai virus, lymphocytic choriomeningitis virus, and rat coronavirus, will not be detected effectively.[36,72,81]

The growth of viruses in cultured cells can be used to quantify the amount of virus produced in an animal.[128] Detection of viral infections by culture is best attempted early in the infection, prior to the production of an immune response against the virus. However, culture-based methods of viral detection are limited by the fact that not all viruses can be grown in cultured cells, and few laboratory animal laboratories are equipped to cultivate viruses. Furthermore, many viruses do not produce visible cytopathic effects in cultured cells or must be passaged multiple times in cultured cells before cytopathic effects are evident, making it difficult to determine if an infection has occurred. Moreover, if a sample is being screened for multiple viruses, several different cell types may be needed to culture all viral species that are present. Once a virus has been isolated from cultured cells, other methods such as serology or PCR are used to speciate the virus.

PCR of viral nucleic acids extracted from infected tissue can detect the presence of a virus far more rapidly than culture-based methods. Culture-based methods of diagnosis only detect infectious forms of the virus, and days or weeks of incubation may be necessary before virus is detectable.[128] In contrast, PCR detects infectious viruses, noninfectious forms of the virus and viral transcripts present in infected cells. For PCR detection, knowing what tissues are infected by the virus is crucial so that the correct sites are sampled.[53] Quantitative PCR (qPCR or qRT-PCR) can be used to measure the amount of viral nucleic acids present in the sample. While the number of viral genomes and the number of infectious virus particles are usually not equal, PCR can be used to compare the viral titers in viral stocks or tissues. PCR is useful for determining the relative titer of viruses that cannot be cultured in vitro (for example, lactate dehydrogenase-elevating virus and murine astrovirus).[22,109,133] During an outbreak, PCR followed by sequencing of the amplified product can be used to determine whether the strain of virus is the same in 2 groups of animals (for example, established colony animals compared with newly imported animals).[25] The ability of PCR to detect viral infections before seroconversion occurs can be used to determine which animals should be removed from the animal room to decrease the risk of viral transmission within a laboratory animal population. For some viruses, such as mouse parvovirus, not all strains of mice are equally susceptible to infection. The duration of infection and amount of virus shed is also variable among strains,[85,87] so testing multiple strains of mice may be necessary to detect the presence of an infectious agent. Thus, multiple rounds of testing are recommended before considering a facility free of an infectious agent.[85]

PCR can be used to detect viruses in animal species for which serological assays are not feasible due to the lack of species-specific secondary antibodies or for which cultured cells suitable for growth and titration of the virus are not available. For example, ranavirus in axolotls (*Ambystoma mexicanum*) and red-eared slider turtles (*Trachemys scripta elegans*)[1,31] and monkey pox in giant pouched rats (*Cricetomys gambianus)* and black-tailed prairie dogs (*Cynomys ludovicianus)*[69] have all been detected using PCR.

Bacterial infections

Traditionally, bacterial infections have been diagnosed by culture in nutrient or selective media under aerobic, microaerophilic, or anaerobic conditions.[24,63] Culturing of bacteria is best performed prior to the administration of antibiotics, and for slow-growing bacteria such as Mycobacteria, can take up to 6 wk. Collection of specimens from sites where normal flora is prevalent may result in overgrowth by normal, nonpathogenic bacteria and may mask the presence of pathogenic bacteria. If a sample is being screened for multiple bacteria, multiple types of culture media and culture conditions may be required. Once a bacterium has been cultured, bacterial smears can be stained to determine bacterial morphology. Panels of enzymatic and fermentation tests or Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF) can be performed on a bacterial isolate to speciate the bacteria. The antibiotic susceptibility of a bacterial isolate can also be determined. Bacterial infections can also be detected in stained tissue sections. For example, *Filobacterium rodentium* (previously: cilia-associated respiratory bacillus) and *Helicobacter* can be visualized in the respiratory or gastrointestinal tract using a silver based stain such as Warthin-Starry.[56,134] Serologic methods are available for the detection of infections with some bacterial species.

Bacterial respiratory infections are difficult to detect using soiled bedding sentinels, as the bacteria usually are not present at sufficient levels in soiled bedding to cause infection and subsequent seroconversion of the sentinel. For example, *F. rodentium* is not effectively detected using soiled bedding sentinels, but can be detected by PCR in frozen or fixed respiratory tissues from infected rodents, or from oral or nasal swabs.[29,30] PCR assays have been developed to detect a wide range of rodent bacterial agents, and PCR can be highly effective at differentiating pathogenic

from closely related nonpathogenic bacterial species. For example, biochemical testing of bacteria cultured from the skin of *C. bovis* infected nude mice may reveal several *Corynebacterium* species with the same biochemical profile, whereas MALDI-TOF or a PCR assay that targets the *rpoB* virulence gene of *C. bovis* will specifically detect *C. bovis*.[37,59]

Not all bacteria can be cultured. Likewise, the different growth rates of the bacteria present in the sample can lead to overgrowth of faster-growing bacterial species, and thus obscure the detection of slower growing bacterial species. In addition, bacteria in the sample that require specialized media or growth conditions may not be detected if the specialized media or growth conditions are not used during screening. An excellent example of this limitation of culture as a diagnostic technique is *Helicobacter*. More than 10 *Helicobacter* species can infect rodents, and rodents can be infected with more than one *Helicobacter* species at the same time.[136] In addition, *Helicobacter* are fastidious bacteria that are difficult to culture. These factors create a challenge when determining the species of *Helicobacter* present in infected animals. In contrast, if PCR is used as the method of diagnosis, generic *Helicobacter* primers that detect most *Helicobacter* species can be used,[122] or primers can be designed to detect only a single species, such as *Helicobacter hepaticus*.[49] Alternatively, multiple sets of primers can be used to detect several species of *Helicobacter* in a single assay (multiplex PCR).[47,110] Multiplex PCR, like other multiplexed assays such as multiplexed fluorometric immunoassays, uses fewer reagents and less sample material than multiple single assays, and is therefore more cost-effective. When generic *Helicobacter* primers are used, the PCR product may then be sequenced to determine the *Helicobacter* species that was amplified. PCR also can be used to rapidly detect other bacteria that grow slowly in bacterial culture media, such as *Mycobacteria* and *Mycoplasma*.[91,98,125] Even for bacteria that can be readily cultured, such as *Rodentibacter*, PCR is more sensitive and more rapid than culturing.[13,112] For zoonotic bacteria, such as *Streptobacillus moniliformis,* using PCR rather than culture-based methods of detection has the additional benefit of decreasing the risk to laboratory personnel.[51]

Parasitic infections

Infestation of rodents with fur mites (*Myobia musculi, Myocoptes musculinus* and *Radfordia affinis*) has traditionally been diagnosed by visualizing adult mites, their larvae and nymphs, or their eggs using microscopy.[3] Because transmission of fur mites via soiled bedding is unreliable, direct sampling of colony mice is recommended.[84] PCR of DNA extracted from fur swabs was more sensitive than fur pluck, tape, and sticky paper tests, and its sensitivity was similar to postmortem pelt exams.[77,121,135] Fur mite PCR can also detect mite DNA at all stages of their life cycle. Because the rRNA genes of *M. musculis* and *R. affinis* are 99% homologous, both mites can be amplified using the same primers.[62] In contrast, primers for *M. musculinus* amplify only *M. musculinus*.[83] PCR of feces has also be used to detect the presence of *M. musculinus* ingested by the mice.[83] The ability to detect *M. musculinus* infestation in a mouse colony using PCR was affected by the age of the mice tested, as older mice had lower egg and mite loads.[121] For optimal detection, the sampling site on the mouse should be considered, as adult *M. musculinus* loads were highest in fur samples from the ventral abdomen and dorsal tail base but egg numbers were highest along the dorsal midline just caudal to the ears.[99] Because PCR detects DNA from both live and dead mites, positive fur swab PCR results from mice treated with antiparasitics can indicate a residual active infection as well as the presence of residual dead mites on the mouse.[121] Follicular mites such as *Demodex musculi* are generally considered to be commensal but can cause disease in immunocompromised mice.[107,130] *D. musculi* has been detected in skin samples from immunocompromised mice using PCR.[108] Antemortem detection of *D. musculi* in immunocompetent mice is challenging, due to the very low mite load present in these animals. Both PCR-based and conventional methods of mite testing can fail to detect *D. musculi* in quarantined mice, leading to the introduction of *D. musculi* into established mouse colonies.[107]

Rodent pinworms (*Aspiculuris tetraptera*, *Syphacia obvelata* and *Syphacia muris)* traditionally have been diagnosed by examination of feces, intestinal contents or perianal tape samples using microscopy; these species can be distinguished morphologically.[3,40] Pinworm PCR is highly specific for differentiating the 3 pinworm species, even when rodents are infected with more than one pinworm species.[26,38,58,60,114] PCR of fecal DNA can also be used to detect *A. tetraptera* eggs and worms. Several studies have compared the sensitivity of fecal *A. tetraptera* PCR to fecal flotation, fecal centrifugation concentration or direct worm detection in intestinal contents, with divergent results.[38,58,60] In some cases, fecal flotation, fecal centrifugation concentration or direct worm detection tests were positive for *A. tetraptera,* while *A. tetraptera* fecal PCR was negative. While hundreds of adult *A. tetraptera* can be present in the intestine of a single infested mouse, and each adult female worm can shed eggs several times during their 21 to 26 d long reproductive lifespan, the number of eggs present in the intestine, and therefore in the feces, can vary widely. Because the number of eggs isolated from feces was highest in 8 to12 wk old mice, testing of feces from young adult mice is recommended when testing a colony for *A. tetraptera*,.[60] PCR of fecal DNA can be used to detect *Syphacia obvelata* or *Syphacia muris* eggs and adult worms.[26,58,114] Fecal *S. obvelata* PCR is more sensitive than either perianal tape tests or direct worm detection from the intestine.[58] Because adult female *S. obvelata* worms migrate to the anus and release their eggs in a single burst, false-negative tape tests can occur if testing occurs between periods of egg release.[127] The intermittent release of eggs can also lead to false-negative pinworm PCR results if feces are collected at times when eggs are not being shed. Repeated collection and testing of fecal samples may be necessary to confirm the presence or absence of pinworm infection. *Syphacia muris*, shows a 2 to 3 wk periodicity of egg shedding, with eggs shed in the highest numbers in midafternoon.[97]

Other intestinal parasites such as *Entamoeba muris, Giardia muris, Spironucleus muris* and *Tritrichomonas muris* can be visualized in intestinal contents. PCR assays for these intestinal parasites have been developed and are routinely performed as part of quarantine procedures and vendor health screens.[6,43,65,73,116]

Fungal infections

In general, fungi grow slowly in culture, and fungal infections often are diagnosed histologically. The most common fungal pathogens present in rodents, *Pneumocystis murina* and *Pneumocystis carinii*, have traditionally been diagnosed histologically from lung samples. [3] PCR of lungs, bronchoalveolar lavages, nasopharyngeal aspirates and oral swabs can be used to detect *Pneumocystis spp*. DNA.[46,70,88,113] *Batrachochytrium dendrobatidis* is a fungal pathogen of high concern in amphibians (anurans, urodeles, and caecilians). Skin swabs from axolotls (*Ambystoma mexicanum*) and rough-skinned newts (*Taricha granulosa*) have been used to detect *B. dendrobatidis* using PCR.[34] Pathogen-specific primers can be used when a particular fungal species is suspected. If a fungal infection is suspected in an animal and

no particular fungal species cultured or identified morphologically, PCR using generic fungal primers and/or genus-specific primers, followed by sequencing of the PCR product, can be used to speciate fungi that are detected.[137]

Biologic contaminant testing

All samples (tumors, cell lines, etc.) to be inoculated into rodents should be tested for the presence of rodent viruses and *M. pulmonis*. Human patient derived xenograft tumors, tissues and stocks intended to be introduced into nude mice should also be tested for *C. bovis*.[95]

The mouse antibody production (MAP) test was developed over 70 y ago and involves the inoculation of the biologic material into mice via several routes (intraperitoneally, intranasally and orally), followed several weeks later by the collection of blood from the mice for serological testing to detect the presence of infectious agents to be excluded.[123] MAP testing requires an area for isolation of the inoculated mice from the rest of the colony.

Once cell-culture based methods were developed for growing most of the agents to be excluded, cell-culture based testing also was used to detect biologic contaminants.[7,12,33,89] This method involves inoculation of several susceptible cell lines with the test substance and then staining of the inoculated cells with antiviral antibodies to determine if the cells have produced viral antigens.

MAP testing and cell-culture methods have now been replaced by PCR/RT-PCR to detect viral and bacterial nucleic acids present in the cultured cells, tissue or /tumor samples. Molecular testing of biologic materials has equivalent or greater sensitivity than traditional MAP testing,[12,14] and PCR-based biologic contaminant testing is faster than mouse or cell-culture based testing. PCR-based testing is also consistent with the principle of the 3Rs in that animals are replaced by an alternative method. PCR can be used to detect agents that cannot be readily grown in cell culture, such as lactate dehydrogenase virus.[19] Furthermore, PCR testing does not result in the production of viruses in cells or animals that can infect rodents or humans; this is particularly important for lethal viruses such as lymphocytic choriomeningitis virus.[9]

PCR of samples collected noninvasively from rodents

Detecting infections using samples collected with noninvasive methods has several advantages. First, it likely provides better animal welfare than does invasive collection. Second, noninvasive collection methods can be performed more frequently than invasive methods without generating animal welfare concerns. Traditionally, performing multiple serological tests required more than 100 uL of blood, which was collected from the retro-orbital sinus of anesthetized mice. This method can be performed weekly if the eyes are alternated. Newer commercial serology methods require less blood, such that unanesthetized mice can be bled from the facial, saphenous or tail vein; however, restraint is needed, and sedation, vasodilation, or shaving may be necessary.[67,120]

In contrast, collection of feces, skin swabs, or fur swabs can be performed daily with gentle manual restraint of the rodent. The ability to collect multiple samples over a short period from the same mouse allows for the time course of agent shedding to be determined.[22,68,78] Sequential samples can be used to determine when a mouse has cleared the infection and therefore is no longer a transmission risk to other colony animals.[21,87] For agents that are shed for only a short period of time (like murine rotavirus) or that are intermittently shed (such as pinworms), infection can be missed if only a single sample is collected. For this type of infectious agent, PCR should be used in conjunction with traditional methods (serology, bacteriology or parasitology). PCR of samples taken from individual colony mice for health monitoring is less cost effective because of the large number of samples that would have to be tested, as compared with serology or PCR based testing of sentinel mice. However, due to the high sensitivity of PCR, up to 10 fecal pellets or swabs can be pooled prior to nucleic acid extraction and still yield a positive result.[93] Therefore, all mice in a cage can be tested as a single sample. The effect of pooling samples on the sensitivity of PCR has not been tested for all agents, and pooling samples could complicate detection of agents that are shed poorly or intermittently. A single fecal sample can yield sufficient nucleic acids to test for a panel of more than 20 rodent intestinal viruses, bacteria, and parasites. Samples can also be pooled for other diagnostic methods (serology, culture, and microscopy).

The use of bacterial 16S rRNA gene primers can result in the amplification of several closely related bacterial species (such as multiple *Helicobacter* species) in fecal samples.[122] In contrast, for detection of a single pathogenic species of bacteria in feces, primers specific for a virulence factor gene are often used. For example, primers specific for the *espB* gene of *Citrobacter rodentium* can be used for PCR testing of mouse feces.[96,124]

Respiratory agents, such as Sendai virus, *F. rodentium* and *Rodentibacter pneumotropicus/Rodentibacter heylii* (previously *Pasteurella pneumotropica* biotypes Jawetz and Heyl[8]) have been difficult to detect using soiled bedding sentinels.[2,32,101,102] Most likely, the amount of these agents that are shed into the bedding and remain infectious is very low. PCR of fecal samples can be used to detect respiratory agents because animals swallow respiratory secretions that contain infectious and noninfectious forms of the agent. However, the sensitivity of detection of respiratory agents in feces is less than that for intestinal agents.[65] Alternatively, minimally invasive oral or nasal swabs can be used to test for respiratory pathogens.[25,52,61,65] A combination of feces, fur swabs, and oral swabs can be used to test quarantined mice for a panel of more than 30 rodent viruses, bacteria, fungi, and parasites.[65] The direct testing of quarantined mice using PCR is therefore consistent with the principle of the 3Rs, in that it eliminates the need for contact sentinels in quarantine and eliminates the production of unwanted litters when imported breeding pairs are housed with female sentinel mice.

PCR of environmental samples

The collection of environmental samples is even less stressful than the collection of feces or fur samples, as it does not require rodent handling. Culturing of infectious agents from environmental surfaces is of limited use, as desiccation drastically decreases the infectivity for many bacteria and viruses.[54] However, samples from environmental sites, including soiled bedding, cage filter tops and individually ventilated cage (IVC) racks, can be collected for PCR testing. One drawback of health monitoring using fecal or fur swab PCR is that it only detects infectious agents that are currently in or on the animal. The use of PCR of environmental samples as part of a health monitoring program allows the detection of agents deposited over a longer period, as each sample represents the accumulation of infectious agents and noninfectious debris deposited into the environment since the last time the sample site was cleaned. Consideration of what sample location to test, how frequently to test, and how

to disinfect the environment if an agent is detected is important when designing a PCR-based environmental monitoring program for infectious agents in research colonies.

Infectious agents shed via multiple routes (fecal, oral, respiratory, skin, fur) can be detected by PCR on cage surfaces and/or in soiled bedding. During an outbreak of rat coronavirus (shed in tears and by sneezing), cage surface swab RT-PCR was more sensitive than RT-PCR of Harderian and salivary glands and could be performed earlier during the infection than serology.[25] During an outbreak of mouse parvovirus (MPV), which is shed in feces, PCR of DNA extracted from swabs of the portion of the cage including the soiled bedding were more effective at detecting MPV in late infection than soiled bedding sentinels, but less effective than pooled fecal PCR.[86] Cage/soiled bedding swabs have also been used to detect fur mites.[74] In cages that contain mice infected/infested with several infectious agents (such as wild mice or those obtained from pet stores), cage swabs represent a single sample that can be tested for multiple agents.[26] Soiled bedding and nesting material can also be tested using PCR.[16,138]

Several methods to monitor the dust present in air exhausted from IVC cages and racks have been developed. These methods can be used to monitor laboratory animal colonies for infectious agents, although differences in the airflow in different models of cages and racks may affect the sensitivity of the method. For IVC rack systems in which the air is filtered as it is exhausted from the cage, testing is best performed at the cage level. Monitoring at the cage level can be accomplished by placing pieces of gauze or filter paper on the underside of the lid of cages of colony or sentinel mice. PCR of nucleic acids extracted from the collection material after 1 to 90 d could detect MHV, MPV, murine norovirus (MNV), Sendai virus, *Helicobacter spp., Rodentibacter spp., M. musculi, M. musculinus, A. tetraptera, R. affinis, S. obvelata, Spironucleus muris* and *E. muris*.[23,29,57] Filters placed on sentinel cage lids for 3 mo, compared with 1 mo, had a higher rate of detection likely because they were exposed to 6 doses of soiled bedding rather than only 2 doses.[39] PCR of DNA extracted from filter top swabs was also effective at detecting fur mites, pinworms and *C. bovis*.[57,95] A recent study showed that filter material placed on the underside of an IVC cage that did not contain sentinel mice but received soiled bedding every other week for 1 to 3 mo was effective at detecting MNV, *Helicobacter spp., Rodentibacter spp., Spironucleus muris* and *E. muris* if the cages were shaken for 15 seconds twice weekly to aerosolize small particles from the soiled bedding.[39]

For IVC rack systems in which the exhaust air is filtered at the rack level, exhaust air dust can be monitored by PCR of filter material placed on the exhaust prefilter, swabs of the exhaust plenum, or filters placed in the exhaust air stream. Several studies have investigated IVC exhaust prefilter testing using several types of IVC racks, durations of filter placement and prevalence of the infectious agents on the IVC rack.[6,23,79,102,138] PCR of nucleic acids extracted from gauze placed on the IVC exhaust prefilter for 1 to 42 d was effective at detecting MHV, MPV, Sendai virus, and *Helicobacter spp.* shed from 4 cages of mice experimentally infected with each agent.[23] PCR of nucleic acids extracted from gauze placed on the IVC exhaust prefilter for 1 to 12 wk detected MHV, MPV, *Helicobacter spp., Rodentibacter spp., E. muris, T. muris, M. musculinus, R. affinis, A. tetraptera* and *S. obvelata,* but not MNV, shed from 1 to 5 cages of mice infected with each agent.[6] PCR of nucleic acids extracted from pieces of gauze placed on the IVC exhaust prefilter for 6 or 12 wk were able to detect several endemic murine viruses and bacteria (MNV, *Rodentibacter spp., Helicobacter sp. Klebsiella oxytoca*, and *Proteus mirabilis*) even when the prevalence of these agents was low in the mice on the rack.[102,138] PCR of nucleic acids extracted from pieces of gauze placed on the IVC exhaust prefilter for 1 or 2 wk were able to detect endemic infection with MNV and *M. musculinus*.[79]

Several studies have investigated using exhaust manifold swabs to monitor exhaust air dust from a variety of IVC racks in which the exhaust air is not filtered at the cage level. Fur mite (*M. musculi* or *R. affinis*) DNA were detected on the horizontal exhaust manifolds of most IVC racks within 2 wk if the rack housed even a single cage of fur mite infested mice.[74] *C. bovis* DNA was detected on the horizontal exhaust manifolds of IVC racks within 1 wk when each rack housed only one cage of *C. bovis* infected mice.[94] *A. tetraptera* DNA was detected in swabs taken from 4 locations in the air handling unit and IVC rack one week after housing multiple cages of pinworm-infested mice.[76] In a recent study, over 1700 IVC exhaust plenum swabs were tested by PCR for 14 opportunistic bacteria and *P. murina; Staphylococcus xylosus, Staphylococcus aureus, R. pneumotropicus, R. heylii, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella oxytoca* and *Klebsiella pneumoniae* were detected in at least 1% of the swabs.[118]

The duration of exhaust air dust exposure needed to detect an agent seems to depend on the amount of infectious agent released into the airflow. A shorter time is necessary if most of the cages on the rack house infected/infested mice or during acute infections when high levels of the agent are being produced. Several IVC rack manufacturers have designed their racks to facilitate exhaust air dust monitoring through the incorporation of specially designed filters and filter holders. A recent study compared 2 of these systems and showed that *Entamoeba spp., Helicobacter spp.* and *Rodentibacter spp.* could be detected with either filter system.[90] In another study, exhaust air dust monitoring using the specially designed filters was more sensitive than soiled bedding sentinels at detecting endemic infections with *Helicobacter spp.* and *Rodentibacter spp.*[92] An essential aspect of using PCR testing of IVC components is complete decontamination of the rack prior to additional testing if an agent is detected, as carryover of nucleic acids can lead to false positive results. False positives can also occur if the primers used will amplify closely related species, such as occurred with the detection of rhabditid nematodes in unautoclaved corncob bedding using Aspiculuris primers.[82]

Any location in the animal facility in which debris generated by laboratory animals accumulates can be tested for infectious agent nucleic acids. PCR of debris collected from the prefilter of an animal bedding disposal cabinet detected several infectious agents endemic in the mouse colony (MNV and *Helicobacter spp.*) and several infectious agents present in a small number of cages of experimentally infected mice (MPV, *M. pulmonis, S. obvelata,* and *M. musculinus*).[26] After vaporized hydrogen peroxide decontamination of a facility that had housed mice infected with *C. bovis*, PCR of swabs of equipment and surfaces in the room and in the adjacent corridors were used to validate the decontamination process.[100] For aquatic species, aquarium water or sediment can be tested by PCR to monitor for DNA of infectious agents. PCR of sump swabs were more sensitive than PCR of zebrafish for detecting mycobacteria.[104] *Pseudoloma neurophilia* DNA was detected in eggs and sperm from zebrafish and from aquarium water if the sample was sonicated to disrupt the spores.[126] PCR testing of filters for water and sediment from aquariums housing zebrafish detected *Mycobacterium spp., Myxidium streisingeri, P. neurophilia* and *Pseudocapillaria tomentosa,* with *Mycobacterium spp.* detected most frequently.[28,103] *Pseudocapillaroides xenopi* was

detected by PCR of sediment from aquariums housing African clawed frogs.[45]

Germ-free Animals and Microbiome Analysis

In addition to routine health monitoring and diagnostic testing of immunocompetent and immunodeficient mice that are colonized with a wide range of commensal organisms, many laboratory animal facilities also maintain colonies of gnotobiotic (including germ-free) animals that require specialized health monitoring. PCR using bacterial 16S rRNA primers, in addition to bacterial culture, are used to confirm the absence of bacteria in germ-free animals.[50] PCR on feces from germ-free mice can reveal traces of bacterial DNA from natural-ingredient based diets, even after the diet is autoclaved, giving the impression that the animal and/or the isolator have been compromised. Therefore, PCR alone should not be used to determine if a biosecurity breach has occurred.

One use of germ-free animals is to study the role of different microbiomes in animal models of disease such as cancer, colitis and diabetes.[64] The microbiome of germ-free animals can be reconstituted with a single bacterial agent or with a complex mix of bacterial agents to study how these agents affect the disease phenotype. Both husbandry (housing, diet, bedding) and animal (genotype, age, sex and source) factors can alter the mouse fecal microbiome.[11,17,20,35,41,42,105,111,115,119,131,132] While microbiome analysis is currently not routinely used as a diagnostic tool in laboratory animal medicine, an understanding of how microbiomes are analyzed and what can alter the microbiome is essential to the field.[27] One method for analyzing the microbiome uses 16S rRNA PCR, followed by sequencing of the pool of PCR products generated.[66] The newer shotgun metagenomics sequencing method does not require PCR, and instead involves breaking the microbial DNA present in the sample into small fragments for sequencing.[75] Bioinformatics analysis of the millions of sequences generated by these methods allows determination of the relative abundance of each microbial species in the population. As sequencing of the complete microbiome (bacteria, viruses, fungi, and parasites) become more affordable, this methodology may replace current diagnostic methods for identifying known infectious agents in rodents and other laboratory animals and has the potential to identify novel disease-causing agents.

Conclusions

PCR has many uses in laboratory animal science and medicine. In some situations, PCR-based testing has replaced other methods, whereas in other cases, using PCR in conjunction with other methods improves the ability to monitor for infectious agents in research animal colonies and their environment. The high specificity of PCR is advantageous in that it can be used to differentiate closely related nonpathogenic and pathogenic strains of an infectious agent. However, small changes in the primer sites of new strains of an agent can lead to false negatives. The second advantage of PCR is its sensitivity (the ability to detect only a few infectious agents); however, this high sensitivity can produce false positives if extraction or PCR reagents become contaminated with low levels of extraneous nucleic acids. Molecular diagnostics are rapid and, when used in the face of an outbreak, can quickly detect which animals are shedding the agent and pose a risk for transmission to other animals in the facility. PCR can be used on a wide range of species, tissue types, excreta and environment samples. An important facet of using PCR is knowing where in or on the animal the infectious agent is most likely to be found and collecting samples from the animal only during active infection. Conversely, PCR testing of environmental samples reveals both current and past infections, as it detects all agents deposited at the sampling site since the site was last sanitized. PCR detects a wide range of infectious agents (viruses, bacteria, fungi, and parasites) and can be used to detect agents that cannot be cultured or that are poorly transmitted to sentinels, such as respiratory agents. The minimal invasiveness of sampling and the reduction in number of animals needed and sampling procedures are all consistent with the ethical principles of the 3Rs.

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