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Overview

Detection of Infectious Agents in Laboratory Rodents: Traditional and Molecular Techniques

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Methods to detect infectious agents in laboratory animals have traditionally been serological and culture based. Molecular methods to detect infectious agents in laboratory animals are being used more routinely. Confusion as to when and how to use molecular methods abounds. In this review, we present a guide to the weaknesses and strengths of using traditional and molecular methods for the detection of infectious agents in laboratory animals.

Molecular methods for the detection of infectious agents are commonly used in human medicine, particularly for diagnosis of HIV, Hepatitis C virus, *Mycobacterium tuberculosis* and *Chlamydia trachomatis* infections (1). Laboratory animal medicine has been a bit slower at routinely using molecular methods for the detection of infectious agents. Several factors contribute to the slow incorporation of molecular diagnostic methods into laboratory animal medicine, including cost and the lack of expertise to perform and interpret molecular tests. This review will outline briefly molecular and traditional methods of infectious disease diagnosis in rodents, their limitations, how to decide which method(s) to use, sample collection and shipment methods, and how to interpret test results. It will not address the use of molecular methods in rodent genetics or infectious disease diagnosis in non-rodent laboratory animals.

Molecular techniques

Molecular methods are aimed at detecting the nucleic acid (DNA or RNA) genome of infectious agents. They can be used at any time during active infection. The specificity of molecular techniques is based on the binding of complementary nucleic acid sequences to each other. The most common molecular methods used to detect infectious agents utilize polymerase chain reaction (PCR) methodology.

PCR involves the rapid and specific amplification of deoxyribonucleic acid (DNA) (2). The first step is the isolation of DNA from the diagnostic sample(s). The complementary strands of the isolated DNA are denatured by heating to 92-95°C. Small single-stranded fragments (16-24 nucleotides) of DNA called primers, specifically designed to bind to the DNA sequence to be amplified, are annealed to the infectious agent genome (DNA template) at 50-60°C. The temperature at which annealing of the primer to the DNA template occurs is dependent on the nucleotide composition and size of the primers selected. After annealing occurs, the temperature of the sample is increased to 72°C, the optimal temperature for activity of the thermostable polymerase. The polymerase synthesizes a new DNA strand by adding nucleotides to the end of the primer. The nucleotides added are the complements of the nucleotides present in the template DNA. During this process, each single-stranded DNA template is amplified two-fold. The original DNA templates and newly synthesized DNA fragments are used as templates in the next cycle of denaturation, annealing and synthesis. The denaturation, annealing and synthesis cycle is repeated 30 to 40 times. After 30 cycles a single DNA template will be amplified approximately 10^9 fold and after 40 cycles a single DNA template will be amplified approximately a 10^{12} fold. A thermocycler can perform these 30-40 cycles in a few hours. The size of the amplified product is determined by the size of the region of DNA between the two primer annealing sites. The PCR product can be visualized under ultraviolet illumination following agarose gel electrophoresis and ethidium bromide staining. Alternatively, if fluorescent tagged nucleotides have been incorporated into the product, the product can be detected using a fluorometer. PCR is highly sensitive due to the exponential amplification of the template DNA, highly specific due to the specificity of the primers and rapid. However, minute amounts of contaminating DNA can lead to false positive results, inhibitors of the thermostable polymerase can lead to false negative results and PCR is relatively expensive.

Reverse transcriptase polymerase chain reaction (RT-PCR) is a variation of PCR which involves the rapid and specific amplification of ribonucleic acid (RNA) (3). RNA is isolated from a diagnostic sample. A primer is annealed to the RNA and reverse transcriptase is used to synthesize a DNA copy of the RNA template. Because most RNAs are polyadenylated (contain a stretch of 50-200 adenosines at their 3' end), an oligo-dT primer can be used to prime the reverse transcription step. The RNA is removed from the DNA copy using RNAse H and the DNA copy is used, with agent-specific primers, for PCR amplification. The advantages and disadvantages of RT-PCR are similar to PCR. Because RNA is more susceptible to degradation than DNA, extra precautions must be used in handling samples for RT-PCR.

Since the development of the PCR assay in 1985, several variations of the basic assay have been developed (4). Nested PCR involves the amplification of a large fragment of a gene followed by reamplification of a smaller internal portion of the gene fragment

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using the initial PCR products as the DNA templates. Nested PCR is more specific than basic PCR because it requires two different sets of primers to bind to the gene and more sensitive because it involves more PCR cycles. Multiplex PCR involves the use of multiple primer sets specific for different infectious agents to generate PCR products of different sizes for each infectious agent.

Hybridization based molecular techniques, while not commonly used in laboratory animal diagnostic laboratories, are often used in laboratory animal-based research. They involve the detection of infectious disease genomes, immobilized on a membrane or within a tissue section, using labeled nucleic acid probes. Southern blots detect infectious agent DNA immobilized on a membrane using a radioactive or enzymatically labeled DNA probe. Northern Blots detect infectious agent RNA immobilized on a membrane using a radioactive or enzymatically labeled DNA probe. In situ hybridization (ISH) detects infectious agent DNA or RNA within cells or tissue sections using a radioactive or enzymatically labeled DNA probe and microscopy.

Serologic techniques

Serologic assays rely on the detection of serum antibodies produced during an infection. Antibodies generally are first detectable 5-7 days post-infection, peak at days 10 to 20 post-infection and last for many months. The specificity of serologic assays is based on the specificity of the antibodies produced for the organism causing the infection. One serum sample can be used to screen for multiple infectious agents. A positive serologic assay does not necessarily indicate an active infection, but rather that exposure to an infectious agent has occurred. Serologic assays rely on the ability of an animal to make antibodies therefore, assays performed on sera from immunodeficient animals may give false negative results.

The enzyme linked-immunosorbent assay (ELISA) is a commonly used serologic test (5). Infected cells, purified bacteria or virus, or recombinant proteins (antigen) are bound to a solid phase (e.g. polystyrene 96 well plate). Sites not occupied by antigen are blocked with an irrelevant protein to prevent non-specific binding of serum antibodies to the solid phase. Diluted test serum is added to the appropriate wells and allowed to bind to the antigen. Unbound antibodies are washed away. An enzyme-linked species-specific anti-immunoglobulin antibody (e.g., alkaline phosphatase linked goat anti-mouse IgG) is added and allowed to bind to the test antibody. Unbound enzyme-linked antibody is washed away. A colorimetric substrate is added and the bound enzyme cleaves the substrate producing a color change that can be quantified with a spectrophotometer. The ELISA is highly sensitive, rapid and inexpensive. However, non-specific cross reactivity between irrelevant antibodies present in the test sera and the antigen used may cause false positive results. This cross-reactivity can be reduced by using highly purified antigens. Modified ELISAs (capture ELISAs), using infectious agent-specific antiserum to "capture" infectious agents, are occasionally used to detect viral infections in rodents within the research laboratory setting.

The indirect fluorescent antibody (IFA) assay is also commonly used to detect infectious agent-specific antibodies (6). Infected cells and uninfected cells are fixed to a microscope slide. Diluted test serum is placed over the cells and allowed to bind to the antigens. Unbound antibodies are washed off the slide. A fluorescent-labeled species-specific anti-immunoglobulin antibody (e.g. fluorescein isothiocyanate labeled goat anti-mouse IgG) is added and allowed to bind to the test antibody. Unbound enzyme-linked antibody is washed off the slide. Specific fluorescence is observed using a fluorescent microscope. Like ELISAs, IFAs are highly sensitive, rapid and inexpensive. Their main weakness is that interpretation is subjective and is highly dependent on the expertise of the observer.

Hemagglutination inhibition (HAI) assays, once a mainstay of serologic testing, are now used to a limited extent (7). The use of this assay is restricted to viruses, which possess proteins (hemagglutinins) on their surface that bind to red blood cells from a specific animal species. Virus is incubated with diluted test serum in a 96 well V-bottom plate. Red blood cells from a specific species are added. If antiviral antibodies have bound to the virus, then the red blood cells will not have agglutinated and will have formed a distinct button in the bottom of the plate. If no antiviral antibodies were present, the virus and red blood cells will have agglutinated and have formed a diffuse network that coats the bottom of the plate. HAI tests lack sensitivity, but are highly specific and can be used to differentiate between closely related viruses such as minute virus of mice (MVM) and mouse parvovirus (MPV). Interpretation of HAI test results is highly subjective, which may complicate definitive diagnosis.

Immunoblot techniques (Western Blots) are not used routinely in laboratory animal diagnostic laboratories, but are used for confirmatory testing (8). Viral or bacterial proteins are separated by size in an acrylamide matrix. The proteins are then transferred from the acrylamide gel to a membrane. Sites on the membrane not occupied by viral or bacterial antigens are blocked with an irrelevant protein. Diluted test serum is added to the membrane and allowed to bind to the antigens. Unbound antibodies are washed away. An enzyme-linked species-specific anti-immunoglobulin antibody (e.g. alkaline phosphatase linked goat anti-mouse IgG) is added and allowed to bind to the sample antibody. Unbound enzyme-linked antibody is washed away. A colorimetric (or luminescent) substrate is added and the bound enzyme cleaves the substrate producing a color change (or burst of luminescence) at the specific positions on the membrane where antigens are bound. Western blots are highly specific, can be used to differentiate between closely related infectious agent species, and to confirm questionable results. Their major drawback is that they are labor and cost intensive.

Immunohistochemistry is a morphologic technique used to detect the presence of infectious agents in cells and tissue sections. Diluted test serum is placed on the cells or tissue section and allowed to bind to the infectious agent antigens. Unbound antibodies are washed off the slide. An enzyme-linked species-specific anti-immunoglobulin antibody (e.g. alkaline-phosphatase-linked goat anti-mouse IgG) is added and allowed to bind to the anti-pathogen antibody. Unbound enzyme-linked antibody is washed off the slide. A colorimetric substrate is added and the bound enzyme cleaves the substrate producing a color change at the specific sites within the tissue where infectious agent antigen is present. Deposits of cleaved substrates are observed using a microscope.

Culture techniques

Culture is the most direct method to detect infectious agents. Tissues, feces, blood, nasopharyngeal washes or environmental samples all can be surveyed using culture techniques. Culture techniques are most effective during the height of an infection, prior to the administration of antibiotics and prior to the production of an immune response. The main limitations of culture tech-

niques are that not all microorganisms grow well in culture and it can take up to 2 weeks to culture and identify bacterial species and even longer to culture and identify a viral agent.

Bacteria can be cultured in nutrient broth or on nutrient plates. Bacterial growth is assessed by increased turbidity in broth cultures or presence of colonies of bacteria on agar medium 1 to 5 days after inoculation. The sample source and the suspected identity of the bacteria will determine the selection of medium (non-selective, selective or differential) and incubation conditions (aerobic or anerobic). For example, because intestinal tissues are colonized with many non-pathogenic commensal bacteria, detection of pathogenic bacteria in the intestine often requires culture on selective media that inhibit growth of commensal bacteria Biochemical tests are usually required to speciate isolates. Antibiotic sensitivity testing of bacterial isolates can be performed to aid in determining treatment regimens.

Because viruses replicate intracellularly, they can be grown only in cultured cells or tissue explants. Sample collection and handling is important, as the infectivity of viruses can be easily destroyed (high temperatures, ice crystals formed during freezing, detergents etc). Viral growth can cause cell death or changes in the cell morphology (referred to as cytopathic effects or CPE), which can be visualized microscopically, 1 to 14 days after inoculation. To screen for a wide range of viruses, several cell types must be inoculated as many viruses grow well only in a single type of cultured cell. Additionally, not all viral infections cause visible changes in the cell and not all viruses grow well in cultured cells. Serological or molecular methods must be used to detect non-cultivable viruses and can be used to speciate cultured viruses.

Uses of molecular diagnostics

Molecular diagnostics have become a part of most laboratory animal quality assurance programs, but confusion still abounds as to when it is most appropriate to use them. The scenarios presented below exemplify the effective use of molecular diagnostics.

Scenario 1: Determining the prevalence and distribution of mouse hepatitis virus (MHV) infection. The initial detection of MHV infection by serology raises several questions: Are the results true or false positives? How far has the infection spread? Is the infection still spreading? The first step is to confirm the serological results using a second test type (e.g. if the original test was an ELISA, the confirmatory test could be an IFA) or alternatively, samples can be sent to a second laboratory for testing. If the initial serologic results are confirmed, then questions of infection prevalence in the initial room and whether the infection spread to mice in other rooms within the facility must be addressed. Since it takes at least 5-7 days for an infected mouse to seroconvert, it is important to remember that serologic tests will indicate the history rather than the current status of the infection. If the mice in the facility are all immunocompetent, then further serologic testing can reveal how many mice were infected in the initial room and whether the infection has spread to mice in other rooms. Serologic testing alone is insufficient in facilities housing mice with known immunodeficiencies, infant mice with immature immune systems, or transgenic mice which may have unidentified immunodeficiencies. Immunocompetent contact sentinels can be used to monitor whether immunocompromised mice are infected with MHV, but this is a time and labor intensive procedure. Alternatively, fecal RT-PCR is an effective technique to determine whether these mice are actively infected with MHV (9,10,11). This strategy can be especially effective in detecting infection of immunocompromised mice since they can shed virus for long periods (8 weeks or longer). Once it has been determined how far the infection has spread the question of whether viral shedding is still occurring must be addressed. Viral culture or RT-PCR can be used for this purpose. However, an adequate number of mice must be tested as MHV usually causes a short-lived infection of less than three weeks duration which may be missed if an insufficient number of mice are tested. Fecal RT-PCR is highly sensitive, rapid and noninvasive and will provide an answer to which mice are still shedding virus. (9). By contrast, culture of MHV is inefficient, time consuming and generally requires euthanasia of the mice for tissue collection.

Scenario 2: Determining if mice are free of Helicobacter infection. *Helicobacter* infection of rodents is a concern in many laboratory animal programs. At least ten *Helicobacter* species of varying pathogenicity have been isolated from rodents. Most laboratory animal programs attempt to exclude *Helicobacter hepaticus* and *Helicobacter bilus* from their colonies because of their documented pathogenicity. Currently there are no commercial serological tests available to the laboratory animal professional and in-house serologic tests generally detect only *Helicobacter hepaticus* (12). Three non-serologic methods exist for *Helicobacter* detection. The Steiner modification of the Warthin-Starry silver stain can be used to detect spiral bacteria in liver sections (13). However, this method is very insensitive, cannot differentiate *Helicobacter* species and is not useful for detection of intestinal *Helicobacter* species. The second method is microbiologic culture of fecal pellets or cecal smears using specialized medium under microaerophilic conditions (14). Because *Helicobacter* species often grow very slowly, inoculated cultures should be held for 2-3 weeks before being considered negative. *Helicobacter* isolates can be speciated using biochemical tests. The preferred method for detection of *Helicobacter* is PCR of tissue or fecal samples. It can be performed in a few hours, is highly sensitive (detects as few as 3 CFU of *Helicobacter*), is highly specific (does not detect other bacterial species) and can be designed to detect one or more *Helicobacte*r species (14,15,16). Fecal *Helicobacter* PCR can be used to determine how effective antibiotic treatments have been at eradicating *Helicobacter* from a colony of mice.

Scenario 3: Testing biological material for viral contamination. Testing of biological materials such as tumors, hybridomas, ascites fluid, embryonic stem cells and continuous cell lines, which are to be inoculated into rodents, for contaminating viruses and mycoplasmas is an essential part of laboratory animal quality assurance programs. Recent outbreaks of mousepox following introduction of cell lines grown in the presence of Ectromelia contaminated mouse serum into mice illustrates the need for this type of testing (17, 18). Traditionally, biological materials to be introduced into mice have been tested for contaminating infectious agents (15 viruses and *Mycoplasma pulmonis*) using the mouse antibody production (MAP) test (19). The MAP test involves inoculation of mice with the biological material. Serum is collected 28 days post-inoculation and is tested for anti-viral or anti-mycoplasmal antibodies using ELISAs or IFAs. This procedure is highly sensitive, but is costly and time-consuming. While *Mycoplasma pulmonis* and some viral agents can be cultured out of biological materials, this method is not very sensitive and is not applicable to viruses which grow poorly in vitro, such as mouse rotavirus. PCR has begun to re-

place the MAP test as the preferred test for detecting viral contaminants in biological materials. A panel of up to 18 PCR and RT-PCR assays can be performed on nucleic acids extracted from biological material. The turnaround time for the PCR panel is several days in contrast to MAP testing, which can take 6 to 8 weeks. A direct comparison of the sensitivity of MAP and PCR-based testing for the detection of 11 murine viruses indicated that PCR-based testing was more sensitive than MAP testing for 8 of the viruses, while detection of the other 3 viruses was comparable in both tests (20, 21). Additionally, the PCR panel is less expensive than MAP testing. MAP testing is renowned for false positive results due to nonspecific reactions of mouse serum in serologic assays. PCR-based testing avoids this problem. Because the infectivity of agents present in the biological material is rapidly destroyed during nucleic acid extraction procedures, the use of PCR decreases the risk of exposure of personnel to zoonotic agents, such as lymphocytic choriomeningitis virus or hantaviruses during testing. Thus, PCR-based testing has significant advantages over traditional MAP testing including increased sensitivity, increased specificity, decreased cost, decreased turnaround times, and decreased risk to personnel.

Scenario 4: Determining whether bacteria isolated from mice are pathogenic or nonpathogenic. The specificity of PCR can be used to differentiate pathogenic species of bacteria from closely related nonpathogenic species. For example, most *Citrobacter* species are nonpathogenic commensal microorganisms whereas *Citrobacter rodentium* causes transmissible murine colonic hyperplasia (22). It is difficult to differentiate *Citrobacter rodentium* from other *Citrobacter* species using microbiological culture techniques. PCR can be used to detect *Citrobacter rodentium* DNA in fecal samples using primers designed to amplify a portion of a virulence gene (eae) found only in pathogenic *Citrobacter*, *Hafnia* and *E. coli*. Restriction enzyme digestion of the PCR product can then be used to differentiate among pathogenic *Citrobacter*, *Hafnia* and *E.coli* species (11).

Scenario 5: Determining the epidemiology of an infection. PCR is very useful in determining the epidemiology of outbreaks. For example, if an infectious agent reappears in your facility shortly after eradication procedures have been completed, it is important to determine whether eradication has failed or whether the agent has been reintroduced into the facility. PCR amplification can be performed on samples collected during the initial outbreak and during the new outbreak and the restriction enzyme patterns or DNA sequences of the PCR products can be compared to determine if the "new" and "old" agent are identical or different.

Sample collection issues

Sample collection is a major concern in diagnostic PCR. Unlike serology, which tests for the presence of antibodies that persist for months, PCR tests for the presence of the infectious agent itself. Therefore, samples used in diagnostic PCR must be selected to maximize the chance of detecting the infectious agent. Sample selection requires adequate knowledge of the pathogenesis of the agent, including tissue tropism and duration of infection. Tissue tropism obviously varies depending on the infectious agent and even for widely disseminated infections, careful selection of tissues for collection is required for optimal results. For example, murine parvoviruses are widely disseminated in many tissues (23, 24); however, optimal tissues for PCR evaluations are mesenteric lymph node or spleen. This may reflect the increased viral load present in these tissues. Tables 1 and 2 provide a list of common rodent infectious agents, the recommended target tissues for PCR evaluations, and information regarding the duration of infection.

Another important factor is the timing of sample collection post-infection. Some infectious agents cause chronic or persistent infections. Correspondingly, PCR evaluations are relatively straightforward for these agents since samples can be collected from animals at various times. Examples of infectious agents that persist in laboratory rodents are *Helicobacter*, the rodent parvoviruses and *Mycoplasma pulmonis*. Other infectious agents are present in tissues for only short intervals before they are effectively cleared by the immune system. Examples of infectious agents that result in acute infections include MHV, Sendai virus and mouse rotavirus (EDIM). For these agents, the timing of sample collection is critical and requires adequate knowledge of their pathogenesis. As a general rule, sample collection 2-4 weeks post-infection is optimal for PCR detection during acute epizootic infections. In endemic infected colonies, especially in breeding colonies, optimal sample collection for PCR diagnostics is typically 2-4 weeks post-weaning since maternal antibodies may preclude infection of young animals.

PCR diagnostics offer the significant advantage of non-invasive antemortem testing. PCR assays using fecal pellets have been developed for several rodent pathogens, including *Helicobacter* species (16), *Clostridium piliforme* and the rodent parvoviruses (21). Assay sensitivity is decreased slightly with fecal samples compared with tissue samples, but the ease of this non-invasive sample collection method outweighs the limited decrease in sensitivity.

The sensitivity of PCR allows for the pooling of samples without loss of sensitivity. For example, fecal pellets from up to 10 rodents can be evaluated with a single PCR assay (16). The ability to pool samples decreases costs, making PCR test costs similar to the cost of serologic testing.

Collection of samples for PCR evaluation

The sensitivity of PCR is its greatest advantage, but it is also one of its greatest disadvantages. Contamination of negative samples with only minute amounts of nucleic acids from a positive sample can result in false positive results. Therefore, strict precautions must be taken to avoid cross-contamination of samples as they are being collected. For instance, instruments that are used in tissue collection or processing must be decontaminated between samples. Commonly used instrument decontamination methods, such as ethanol treatment and subsequent flaming, are ineffective since inactivation of the infectious agent does not necessarily eliminate nucleic acids that can then be amplified by PCR. A more effective approach for decontamination of instruments is to soak used instruments in a 1:10 dilution of bleach for 5 min. The bleach effectively destroys nucleic acids; thus, preventing cross-contamination of samples.

Storage and shipment of samples for PCR evaluation

Storage of samples for PCR evaluation also is a critical issue. Samples should be frozen immediately after collection in sterile tubes. When testing for bacteria and DNA viruses, tissues should be frozen in a -20°C freezer until processed. For long-term storage of tissues, a unit that does NOT self-defrost should be used. The repeated warming cycles that prevent frost buildup inside a

Table 1. Viruses and target tissues for PCR evaluations

Virus	Genome	Tissue(s) for PCR evaluation
Adenoviruses	DNA	
Mouse adenovirus 1 (MAD1)		Lung
Mouse adenovirus 2 (MAD2)		Intestine
Guinea pig adenovirus (GpAD)		Lung
Cytomegaloviruses*	DNA	
Mouse cytomegalovirus (mCMV)		Spleen, salivary gland
Rat cytomegalovirus (rCMV)		Spleen, salivary gland
Guinea pig cytomegalovirus		Spleen, salivary gland
Ectromelia	DNA	Spleen, skin lesions
K virus	DNA	Kidney
Mouse thymic virus (MTV)*	DNA	Salivary gland
Parvoviruses*	DNA	
Mice minute Virus (MMV, formerly MVM)		Mesenteric lymph node, spleen, kidney, intestine
Mouse parvovirus (MPV)		Mesenteric lymph node, spleen, kidney, intestine
Kilham's rat virus (KRV)		Mesenteric lymph node, spleen, kidney, intestine
H-1		Mesenteric lymph node, spleen, kidney, intestine
Rat parvovirus (RPV)		Mesenteric lymph node, spleen, kidney, intestine
Polyoma virus	DNA	Skin, mammary gland
Coronaviruses	RNA	
Mouse hepatitis virus (MHV)		Feces, lung
Rat Coronavirus (RCV/SDAV)		Lung, Harderian gland, salivary gland
Hantaviruses	RNA	
Hantaan		Kidney
Seoul		Kidney
Puumala		Kidney
Sin Nombre		Lung
Lactate dehydrogenase virus (LDV)*	RNA	Spleen
Lymphocytic choriomeningtis virus (LCMV)*	RNA	Kidney
Pneumonia virus of mice (PVM)	RNA	Trachea, lung
Reovirus 3	RNA	Liver, lung, feces
Rotavirus (EDIM)	RNA	Feces, intestine
Sendai virus	RNA	Trachea, lung
Theiler's meningoencephalitis virus (TMEV)*	RNA	Feces, intestine

* Persistence documented.

Table 2. Bacteria, protozoa, and fungi and target tissues for PCR evaluations

Organism	Tissue(s) for PCR evaluation
Cilia-associated respiratory bacillus*	Nasopharynx, lung
Clostridium piliforme	Cecum, feces
Citrobacter rodentium	Feces, cecum
*Corynebacterium bovis**	Skin scrapings, skin
Encephalitozoon cuniculi	Urine, kidney, brain
*Helicobacter** *(genus-specific)*	Cecum, feces
H. bilis (species-specific)	Cecum, feces
H. hepaticus (species-specific)	Cecum, feces
H. typhlonius (species-specific)	Cecum, feces
H. rodentium (species-specific)	Cecum, feces
Mycoplasma (all known species)	
*Mycoplasma pulmonis**	Trachea, nasopharynx
Pasteurella sp. (all known species)	
*Pasteurella pneumotropica**	Trachea, nasopharynx, conjunctiva
*Pneumocystic carinii**	Lung
*Proteus sp.**	Feces, cecum
*Pseudomonas aeruginosa**	Feces, cecum
*Salmonella sp.**	Feces, cecum
*Staphyloccus aureus**	Nasopharynx

*Persistence documented.

self-defrost freezer are detrimental to DNA integrity. When testing for RNA viruses, samples should be frozen as quickly as possible, ideally in a dry ice/alcohol bath or in liquid nitrogen, to prevent RNA degradation by RNases. After freezing, samples should be held at -80°C or lower. Biological materials including ascites fluid, hybridomas, purified monoclonal antibodies, mammalian cells, and frozen stocks of cell lines that may contain cryoprotectants such as glycerol or dimethylsulfoxide can be tested by PCR-based assays. Biological materials are generally tested for both DNA and RNA viruses therefore should be frozen as quickly as possible, ideally in a dry ice/alcohol bath or in liquid nitrogenand be stored at -80°C. Samples to be shipped to a diagnostic laboratory for evaluation, should be shipped using an overnight courier and shipping containers containing a dry ice sufficient to keep samples frozen for 48 h. Deviations from these storage and shipping recommendations may result in false negative results.

One exception to the general recommendations for sample storage and shipment is sample storage and shipment of feces that are to be evaluated for the presence of *Helicobacter* species. Fecal pellets can be collected from the bedding of *Helicobacter* infected mice within 48 h after a bedding change (16). *Helicobacter* DNA is stable in feces at room temperature for at least one week post-collection, but if pellets are to be held for several days before PCR testing, it is advisable to freeze the samples at -20°C. Because of the stability of the *Helicobacter* DNA, fecal pellets can be shipped at ambient temperature.

Sensitivity and specificity of PCR testing

PCR is touted for its outstanding sensitivity and specificity which allows for the detection of minute levels of infectious agents. In general, the sensitivity of viral PCR assays is 1-10 virions, while bacterial PCR assays are capable of detecting as few as 3-10 bacteria (11,21).

The sensitivity of PCR assays is affected by several factors, including inhibitors of thermostable polymerases present in the sample, the quality of the DNA or RNA template, and the amplification conditions. Polymerase inhibitors are a major concern since they reduce the sensitivity of the assay and can lead to false negative results. A number of compounds cause inhibition of thermostable polymerases, including complex polysaccharides, hemoglobin and its metabolic products, immunoglobulin G and lactoferrin (25, 26, 27, 28, 29). While polymerase inhibitors are present in many types of specimens, blood and feces are especially problematic. Two approaches are used to minimize inhibitor effects: 1) removal of inhibitors during nucleic acid purification, and 2) dilution of extracted nucleic acids. Commercial products are available for purification of DNA and RNA free of polymerase inhibitors. These products should be carefully compared by laboratories doing diagnostic testing to determine which product is optimal for samples being tested in their facility. Elimination of inhibitors by dilution of nucleic acid samples can be considered, but at high dilutions target DNA concentrations may be below the sensitivity of the PCR assay and lead to false negative results. To assess whether polymerase inhibitors are present, purified DNA or RNA from the specimen is spiked with DNA from a known positive control. If the amount of PCR product generated from the positive control decreases or no product is detected in the presence of specimen DNA, one can conclude that the specimen contains polymerase inhibitors.

The quality of the extracted DNA or RNA is an important key to obtaining valid PCR results. Specimens and purified nucleic acids must be collected and stored appropriately. As mentioned above, DNA samples should be stored at -20°C in a non-self-defrosting freezer and RNA samples should be stored at -80°C to prevent nucleic acid degradation.

Sensitivity is also governed by reaction conditions, e.g. salt, $MgCl_2$, primer concentrations, the type and amount of polymerase utilized. Optimal concentrations of each of these components must be determined for each assay. Assay sensitivity is also influenced by the number of amplification cycles performed. In general, the greater the number of cycles, up to a maximum of 45-50 cycles, the more sensitive the assay is. Beyond 45-50 cycles, polymerase activity is generally diminished to the point that further amplification is negligible.

The specificity of PCR testing is controlled primarily by the primers used and the annealing temperature. The greatest specificity of PCR occurs when the primers match the target template exactly and the annealing temperature is high enough to prevent binding of the primers to the template in the absence of a perfect template-primer match.

Interpretation of PCR results

PCR results must be interpreted carefully. No test is 100% sensitive or specific and therefore results should always be confirmed, particularly positive results. Confirmation can be obtained by testing additional specimens with the same assay or by testing the original specimens by another diagnostic assay. For example, a positive MHV RT-PCR result may be confirmed by RT-PCR of additional specimens from cohort animals, by serologic evaluation of the initial animals, or by a RT-PCR assay that uses different primers. Decisions should not be made without confirmatory testing.

It is also important to recognize that PCR-positive animals may not be seropositive. Antibody responses may not have developed yet (e.g. early in infection) or an animal may not be capable of generating a humoral antibody response (e.g. *nu/nu* or *scid* mice). Similarly, transgenic or knockout mice may be unexpectedly immunodeficient if genetic manipulation has disrupted immune function.

Interlaboratory variability of PCR testing

Several factors may account for differences in testing results between laboratories. Primer sets and amplification conditions differ between laboratories. The sensitivity and specificity of primer sets and amplification conditions vary depending on the isolate or strain of the infectious agent being amplified. For instance, Laboratory A's PCR assay may have a higher sensitivity for detection of the JHM strain of MHV than does Laboratory B's, but the latter's assay may have a higher sensitivity for detection of the S strain of MHV. Thus, comparison of test results among testing laboratories can be complicated by the lack of standardization of assays. A similar concern affects serologic testing, as serology laboratories often produce their own antigens and utilize laboratory-specific protocols for serologic evaluations. For both serologic and molecular diagnostic testing, variation in test results can be viewed as a weakness, but it also can be viewed as a strength because it encourages confirmation of critical results by a different laboratory using a different assay. Importantly, as with any other type of assay, discrepancies in PCR results may be due to human error, reagent failure or equipment malfunction.

In this context, laboratories with sound quality control programs should be sought for diagnostic testing. One approach to minimizing differences in results among diagnostic testing laboratories is a performance assessment program utilizing well-characterized specimens. A program has been proposed by several diagnostic laboratories in the United States that will distribute specimens for evaluation by PCR and RT-PCR assays. This program will allow participating laboratories to assess the quality of their molecular diagnostic assays and to address problems in sensitivity or specificity.

Conclusion

In summary, several issues related to the use of traditional and molecular methods to detect microbial pathogens have been reviewed. Molecular diagnostic techniques are becoming an integral part of laboratory animal quality assurance programs, supplementing traditional serology, bacteriology, virology, and pathology techniques. Because traditional and molecular methods have different strengths and weaknesses, no one method can be used for all testing. Collective testing using diverse methods is crucial for the accurate detection and staging of infections. The scenarios presented demonstrate that adequate knowledge about the pathogenesis of an infectious agent is required to develop a sound approach to PCR based detection of the agent. It is important to remember that a little knowledge can be disadvantageous, so seek help if you are unsure what samples to collect, what tests to run or how to interpret results.

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