Molecular Diagnostic Assays for Infectious Diseases in Cats

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Numerous options are available for the diagnosis of infectious diseases in feline medicine. Historically, cytologic techniques, histopathologic techniques, and microbiological cultures are used for the demonstration of the presence of the organism and serologic antibody titers for the demonstration of immune response to an infection. However, these techniques have inherent deficiencies. Cytologic and histopathologic techniques require the organism to be large enough to be seen microscopically and in sufficient numbers for visualization. The sensitivity of organism visualization for diagnosis often decreases as disease progresses because the host's immune response decreases the number of organisms in the body. Microbiological culture requires specific knowledge of the organism's requirements for growth and may require specific handling for organism preservation and culture periods longer than are clinically useful. Immune response to an organism, as demonstrated by serum antibody titers, can be sensitive but requires days to weeks for a host response and demonstrates only exposure to the organism and not the disease secondary to the organism or even the current infection.

For a diagnostic test to be practical, it must be useful (high sensitivity and specificity), reliable (reproducibility), convenient, and cost-effective. For these reasons, the use of molecular assays in feline medicine has gained favor for the diagnosis of diseases caused by organisms that are difficult to be identified, detected, or cultured in a timely fashion. Because most veterinarians rely on the proper use of molecular assays on a daily basis to practice high-quality veterinary medicine, this article provides a brief overview of the technologies available, their shortcomings and advantages, and the current clinical applications of the technologies in feline medicine.

Molecular assays rely on the detection of the nucleic acids DNA and RNA. These nucleic acids are a part of the genetic makeup of the organism and consist

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of 4 nucleotides in varying sequences. Many portions of DNA and RNA are highly conserved between organisms, whereas other portions are specific to the organism on a family, genus, species, or even strain level. The sequence specificity is used to detect the organisms within clinical samples, using some form of complementary sequence and sometimes a signaling molecule. Signaling molecules are often some form of a fluorescent molecule to improve sensitivity.

DETECTION OF PATHOGENS WITHOUT AMPLIFICATION

The simplest application of molecular tools for the detection of infectious organisms is the use of a complementary nucleic acid sequence, termed a probe, which has been tagged with a fluorescent molecule. This probe is then added directly to a clinical sample, either a fluid or tissue section. Multiple probes, with different fluorescent tags, can be added to a single sample, allowing for the detection of several organisms in a single assay. This technique of hybridization of a probe to a target sequence in an organism was one of the first applied techniques in human clinical medicine but has not gained widespread use in feline medicine. This technique is still used routinely to monitor the viral load in patients infected with human immunodeficiency virus undergoing antiviral therapy. The feline therapeutic correlate, treatment of feline immunodeficiency virus (FIV), has not advanced to as finely tuned a protocol. Probe hybridization is rapid, user friendly, and simple to perform. This technique also removes the need for specialized culture conditions, but sensitivity of this technique is poor compared with other molecular techniques. Prior enrichment of the sample via microbiological culture improves sensitivity but increases the time needed for the assay and requires knowledge of the microbiological cultural demands of the organisms, eliminating many of the advantages of the technique for clinical application. This technique remains useful for the detection of slow-growing organisms, such as fungi and mycobacteria, in the presence of other more rapidly growing organisms in culture and for the rapid quantification of the organism load in a nonenriched clinical sample.

A more specialized application of probe hybridization is in situ hybridization. This technique uses the same theory as the simple probe hybridization but applies it to tissue samples, allowing the detection of the organisms of interest in association with inflammatory lesions or specific areas of tissue. This technique is useful in situations in which a large number of organisms can be detected, but the organisms may be part of a normal flora, such as those in the gastrointestinal tract. In situ hybridization allows the user to determine if certain bacterial species are associated with inflammation or are beyond the superficial layers of the gastrointestinal tract. Fluorescent molecules are the most common signaling mechanism used, and in this case, the method is abbreviated FISH (fluorescent in situ hybridization). The technology is as simple as a solution-based probe hybridization but requires skilled operators because nonspecific background staining can cause false-positive results.

DETECTION OF PATHOGENS WITH AMPLIFICATION: POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was first described in 1985[1] by Kary Mullis and colleagues, for which Mullis later received the Nobel Prize in Chemistry. This powerful tool uses the cyclic amplification of a strand of DNA using a proprietary enzyme to produce an exponential number of identical copies to a detectable level (**Fig. 1**A). The DNA is then analyzed, usually on a gel, to determine if it is of the predicted size for the reaction (see **Fig. 1**B). Application of this technique allows for the detection of minute numbers of organisms in a very small sample, an advantage in feline
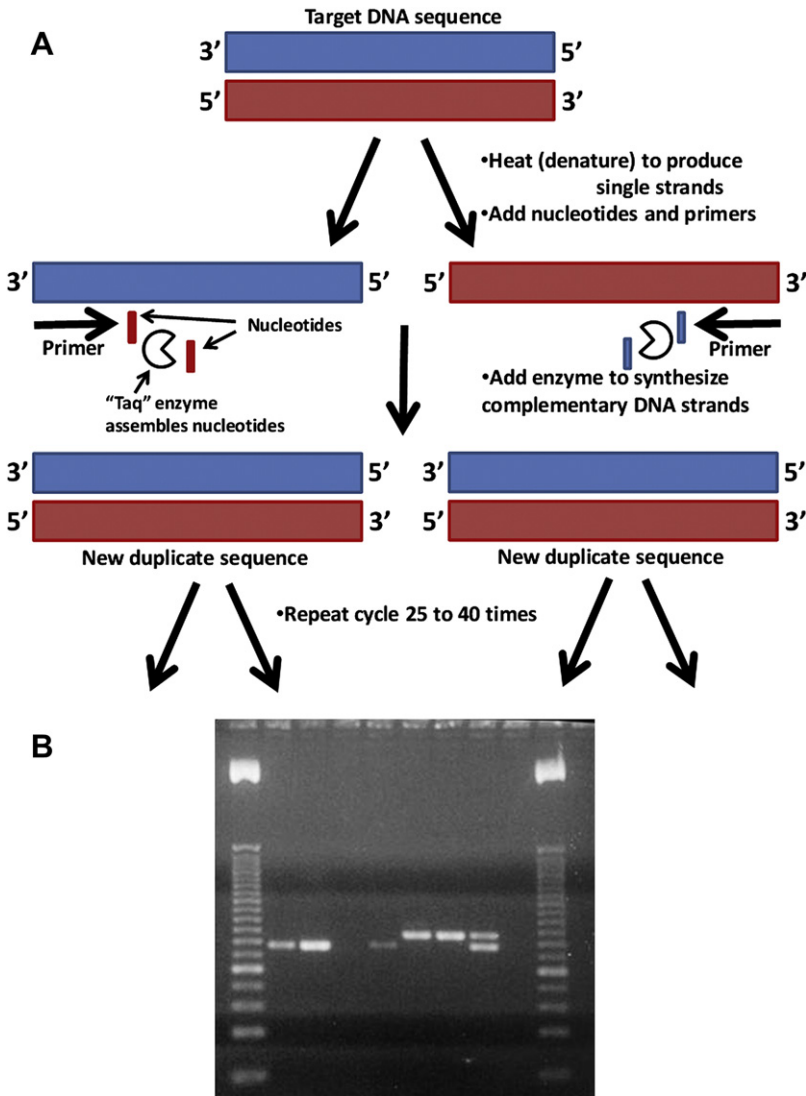
Fig. 1. (*A*) PCR. Short sequences of nucleotides called primers are annealed to the target DNA after the separation of the double strands. A proprietary enzyme is used to produce complementary strands of DNA during the synthesis step. Denaturation is repeated, and replication of the newly formed DNA strands, as well as the original target DNA, is repeated. (*B*) The DNA produced in the reaction (described in [*A*]) is then visualized using gel electrophoresis. The size of the product is compared with a standard to confirm that the predicted product has been produced.

medicine given the size of patients. PCR is superior to probe hybridization techniques in sensitivity because of this amplification. Although the exponential amplification of the original target provides the greatest advantage of this technique, it is also the basis of the greatest downfall, contamination. Initially, PCR was restricted to highly specialized research and diagnostic laboratories. Commercially available kit-based

technology now allows for more widespread use of PCR. This technology has decreased cost and improved availability but increases concerns regarding quality control. Strict adherence to good laboratory practice must be observed for credible results. This criterion raises a problem for clinicians because they cannot be aware of the actual laboratory practices of the laboratory supplying the assay. Therefore, it is recommended that if a recently published PCR assay is to be used clinically, the originating laboratory be used if at all possible because the laboratory personnel are familiar with the nuances of the individual assay and have experience with the largest number of clinical samples.

DNA of inactivated organisms injected into the bloodstream of laboratory animals has been detected more than a week after injection, demonstrating not only the high sensitivity of the technique but also the care that must be taken in interpreting results. Detection of an organism's nucleic acid in the bloodstream does not necessarily mean active infection or disease. The presence of nucleic acid simply indicates that the nucleic material of the organism exists in the host and not that the organism is alive, capable of replication, or actually causing clinical signs in the host. Correlation with clinical signs of a known syndrome associated with the organism and/or a response to therapy must be used in conjunction with the results of PCR. Finally, to prevent false-negative results, samples tested should be obtained before treatment because the treatment may decrease the organism load below the level of detection of even PCR, even though the organism is still present in the host.

PCR: VARIATIONS ON A THEME

Because of the structural differences between RNA and DNA, the enzyme used in PCR can only duplicate strands of DNA. However, many infectious agents are RNA viruses. Therefore, a preliminary step, reverse transcription (RT), to create a complementary strand of DNA from the target RNA must be performed. Amplification of the complementary DNA via PCR is then performed; this method is commonly known as RT-PCR.

The primers used in PCR can be designed to amplify the nucleic acids of only members of a certain genus, species, or even strain. The detection of suspected organisms is by far the most common use of PCR in veterinary medicine. When a single organism is targeted in an assay, the technique is termed a singleplex PCR. If multiple targets can be detected in a single assay, the technique is termed a multiplex assay. It is clearly most advantageous to investigate the presence of multiple organisms in a single assay. However, in the PCR assay, each target sequence competes with each other for the common building blocks that allow the reaction to proceed: the enzyme, nucleotide, and various buffers and ions. Therefore, multiplex reactions are frequently less sensitive than singleplex assays and require extensive optimization to be useful.

When no specific organism is identified as a likely cause of clinical signs, the use of broad-range or degenerate primers that amplify the DNA of the members of an entire genus or even kingdom can be used, targeting highly conserved regions of the nucleic acids. The most common application of PCR is for the rapid detection and identification of bacteria or fungi in clinical samples.[2,3] The PCR results can be available in as early as 2 hours and provides information on whether fungal or bacterial nucleic acids are present in the sample. Subsequent analysis of the PCR product may then be used to identify the infecting organism much more rapidly than traditional microbiological techniques and may be more sensitive for the detection of fastidious organisms. However, antimicrobial sensitivity is not available while using this technique; therefore, PCR is complementary to traditional culture techniques. However, the use of PCR for

the detection of certain genes that encode for antimicrobial resistance is also starting to gain clinical use and may provide additional rapid information before antimicrobial sensitivity results are being available.[4]

The most recent application of PCR in clinical feline medicine has been real-time quantitative PCR (qPCR). Quantification by traditional endpoint PCR is difficult because after so many amplification cycles, most samples yield essentially the same amount of product because some limiting reagent would have been completely consumed before the final amplification cycles. In 1992, Higuchi and colleagues[5] reported a technique for monitoring the production of DNA during each amplification cycle so that the original quantity could be extrapolated by the identification of the logarithmic amplification phase of each individual reaction. This technique uses fluorescent dyes or probes that produce a signal after formation of the product (**Fig. 2**). During each amplification cycle, a detector records the amount of fluorescence in the sample. Gene expression is commonly measured using qPCR and has been used in many disease states in felines to evaluate host response to an infection.[6–12] Pathogen detection and load determination are some of the many applications of this technology. This assay has all the advantages of traditional endpoint PCR (sensitivity, specificity), offers a more rapid result, and has the ability to quantitate microbial DNA or RNA load. However, with these improvements additional concerns regarding quality control have been added. The fluorescent dyes and probes used to detect the PCR product allows for even more sensitive assays and susceptibility to contamination leading to false-positive results. Accuracy of quantitation is reliant on the availability of a reproducible high-quality standard curve. In an attempt to regulate this rapidly expanding field, minimum laboratory standards have been proposed.[13] Although these guidelines can be used to evaluate the quality of a published protocol, many diagnostic laboratories use proprietary reactions that are not subject to peer review. But because of this practice, the practitioner needs to request the evaluation data from the diagnostic laboratory to evaluate the clinical utility of the assay until it has been evaluated in a peer-reviewed journal.

MOLECULAR ASSAYS IN FELINE MEDICINE: CURRENT APPLICATIONS

The following is a review of assays that are currently commercially available in feline medicine for the diagnosis of infectious diseases. It is anticipated that many more applications will be developed in the upcoming years, and it is the responsibility of the clinician to maintain the knowledge of the current literature to apply these new assays in an appropriate manner. Molecular assays simply indicate the presence of a microbial DNA or RNA and not that of the disease. The ability of an assay to detect an organism is measured by its sensitivity and specificity: the frequency at which an assay can detect an organism (sensitivity) and not other organisms (specificity). The true measure of a test for disease diagnosis is the predictive value. Positive predictive value (PPV) is the measure of a test's ability to predict the presence of disease, and negative predictive value (NPV) is the ability of an assay to predict the absence of disease. However, most diagnostic laboratories can report only sensitivity and specificity because they are easier to calculate, and hence the onus of remembering the predictive values of an assay for the syndrome being assessed is on the clinician.

Respiratory Agents

Feline calicivirus (FCV) infection is a common differential diagnosis in cats with clinical evidence of rhinitis and stomatitis. Less commonly, FCV infection is associated with conjunctivitis, polyarthritis, and lower airway disease in kittens. Virus isolation can
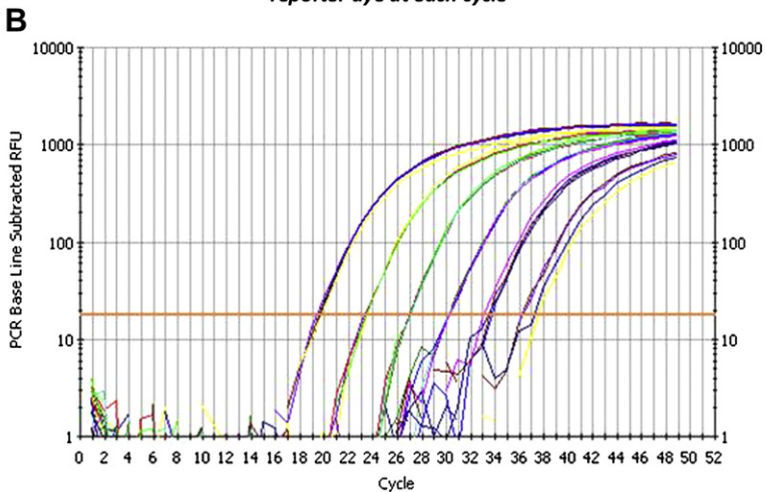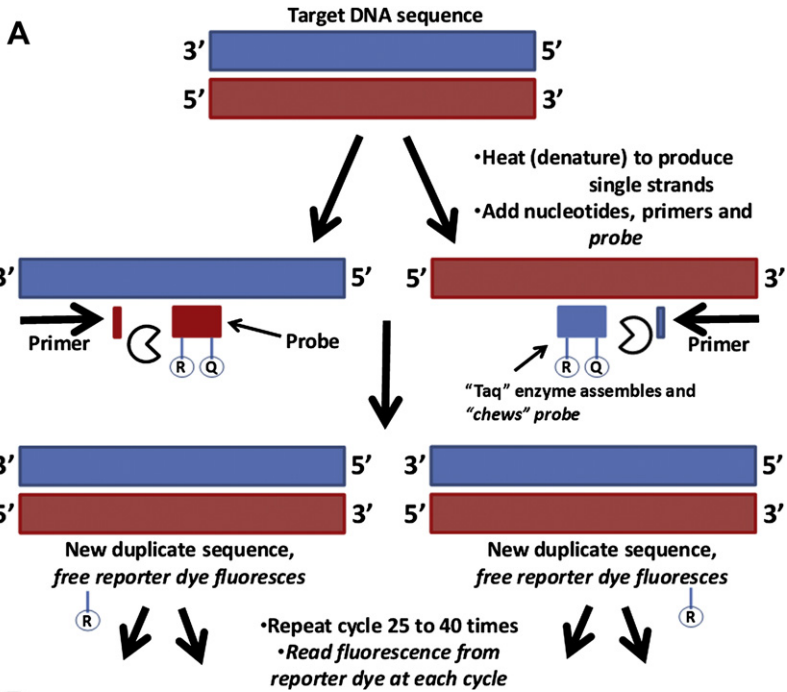
Fig. 2. (*A*) qPCR. In the most commonly used chemistry, the standard PCR assay is enhanced by using a fluorescent probe that fluoresces only after the removal of a quencher dye in close proximity to the reporter dye. The quencher dye is removed by the enzyme that synthesizes new strands of DNA as in traditional PCR. At each step, fluorescence is measured, allowing for the extrapolation of the amount of product present during each replication phase. (*B*) The change in fluorescence is then plotted against time (number of cycles), and a starting quantity can be calculated by the extrapolation of the signal produced during the exponential replication phase.

be used to document current infection but it takes at least several days for results to return. Because of widespread exposure and vaccination, the PPV of serologic tests is poor. RT-PCR assays can be used to amplify the RNA of FCV, and the results can be returned quickly. However, these assays also amplify vaccine strains of FCV. FCV RNA can be amplified from samples collected from normal carrier cats as well as from clinically ill cats, and so detection of FCV RNA has a poor PPV. For example, in one study, the presence of FCV RNA failed to correlate to the presence or absence of stomatitis in cats.[14] In addition, amplification of FCV RNA cannot be used to prove virulent systemic calicivirus infection. Results of FCV RT-PCR can also be false negative and so can have poor NPV.

Infection with feline herpesvirus 1 (FHV-1) is a common differential diagnosis in cats with clinical evidence of rhinitis, stomatitis, conjunctivitis, keratitis, and facial dermatitis. Because of widespread exposure and vaccination, the PPV of serologic tests is poor. FHV-1 can be documented by direct fluorescent staining of conjunctival scrapings, virus isolation, or PCR. FHV-1 DNA can be amplified from conjunctiva, nasal discharges, and pharynx of healthy cats, and so the PPV of conventional PCR assays is low.[15] Currently used PCR assays also detect vaccine strains of FHV-1, further lessening the PPV of the assays.[16] In one study, the presence of FHV-1 DNA failed to correlate to the presence or absence of stomatitis in cats.[14] In one study, results of qPCR may ultimately prove to correlate to the presence or absence of the disease but have failed to correlate to the presence of conjunctivitis.[17] The NPV of FHV-1 PCR assays is also in question because many cats that are likely to have FHV-1–associated disease show negative results. These results may relate to the clearance of FHV-1 DNA from tissues by a hypersensitivity reaction. Tissue biopsies have greater sensitivity than conjunctival swabs but do not necessarily have a greater predictive value. FHV-1 DNA can be amplified from the aqueous humor of some cats but whether this amplification indicates FHV-1–associated uveitis is unknown.

Mycoplasma spp, *Chlamydophila felis*, and *Bordetella bronchiseptica* are other common respiratory pathogens in cats. As for FHV-1 and FCV, PCR-positive test results cannot be used to distinguish a carrier from a clinically ill cat. In addition, PCR assays do not provide antimicrobial drug susceptibility testing, and so for cats with potential bordetellosis, culture and sensitivity is the optimal diagnostic technique, especially in case of an outbreak. *Toxoplasma gondii* DNA has been amplified from the airway washings of some cats with lower respiratory tract disease, and so PCR is an option for evaluation of samples from diseased animals from which the organism is not identified cytologically.

Gastrointestinal Agents

The detection of *Giardia* spp is generally made with the combination of fecal flotation techniques and wet-mount examination. Fecal antigen tests are also accurate, and there are several assays available for point-of-care use, including one labeled for veterinary use.[18] Fecal PCR assays often show false-negative results because of PCR inhibitors in stool, and so PCR should not be used as a screening procedure for this agent. However, *Giardia* spp PCR assays can be used to determine whether the infective species is a zoonotic assemblage, which is the primary indication for this technique. However, it now seems that assemblage determination should be performed on more than 1 gene for most accurate results.

Although *Cryptosporidium* spp infection is common, it is unusual to find *Cryptosporidium felis* oocysts using fecal flotation in cats. Acid-fast staining of a thin fecal smear is cumbersome and insensitive. Antigen assays titrated for use with human feces are inaccurate when used with cat feces. Thus, PCR may aid in the diagnosis of

cryptosporidiosis in dogs and cats and has been shown to be more sensitive than immunofluorescence assays (IFAs) in cats.[19] *Cryptosporidium* spp PCR assays are indicated in IFA-negative cats with unexplained small bowel diarrhea and when the genotype of *Cryptosporidium* is to be determined. However, *Cryptosporidium felis* infection in cats is common, and so positive test results do not always prove that *Cryptosporidium felis* is the cause of the clinical disease. No drug is known to eliminate *Cryptosporidium* spp infections and small animal strains are not considered significant zoonotic agents; so PCR is not currently indicated in healthy animals. PCR assays are also available for the detection of DNA of *Tritrichomonas foetus*, *Salmonella* spp, *Campylobacter* spp, *Clostridium* spp, parvoviruses, and *T gondii*, and RT-PCR assay is available for coronaviruses. Trophozoites of *T foetus* can often be detected on wet-mount examination of fresh feces, which can be completed as an in-clinic test. The DNA of *T foetus* can be detected in healthy carrier cats, and so positive test results do not always prove illness from the organism.[20] In cases with suspected salmonellosis or campylobacteriosis, assessment should be done by culture rather than by PCR to determine the antimicrobial susceptibility patterns. In dogs, the PPV of *Clostridium* spp PCR assays on feces is low, and if the assay is used, it should be combined with enterotoxin assays. Information in cats is currently lacking. At present, there is noevidence that parvovirus PCR assays on feces is superior to currently available antigen assays and that currently used PCR assays for panleukopenia virus amplify vaccine strains. Oocysts of *T gondii* are shed only for about 7 to 10 days, and millions of oocysts are generally shed during this period, making the organism very easy to identify. Thus, PCR assays are usually not needed to diagnose this infection. Because virus isolation is not clinically practical, RT-PCR is used most frequently to detect coronavirus RNA in feces. However, positive test results do not differentiate feline infectious peritonitis (FIP)-inducing strains from enteric coronaviruses.

Blood-Borne Agents

Mycoplasma haemofelis (Mhf), Candidatus *Mycoplasma haemominutum* (Mhm), and Candidatus *Mycoplasma turicensis* (Mtc) can be found in cats. In at least 2 studies of experimentally infected cats, Mhf was found to be apparently more pathogenic than Mhm. It seems that Mtc has intermediate pathogenicity. Diagnosis is based on demonstration of the organism on the surface of erythrocytes on examination of a thin blood film or PCR assay. The number of organisms fluctuates, and so blood film examination results can be false negative up to 50% of the time. It may be difficult to find the agent cytologically, particularly in the chronic phase. Thus, PCR assays are the tests of choice because of their sensitivity.[21] Primers that can amplify the DNA of all the 3 hemoplasmas are available. qPCR assays can be used to monitor copy numbers during and after treatment but do not have greater sensitivity, specificity, or predictive value than conventional PCR assays.[22] PCR assays should be considered in the evaluation of cats with unexplained fever or anemia and that are cytologically negative for the hemoplasmas. In addition, the American College of Veterinary Internal Medicine recommends screening cats for hemoplasmas by PCR assays for their use as blood donors.[23] Many cats are carriers of the relatively nonpathogenic Candidatus Mhm, and so positive test results may not always correlate to the presence of the disease (poor PPV).

Cats can be infected by an *Ehrlichia canis*–like organism[24] and *Anaplasma phagocytophilum*.[25] Little is known about the other agents in these genera in regard to cats. Because the organisms are in different genera, serologic cross-reactivity is variable. Thus, although the clinical syndromes can be similar, there is neither a single serologic test to document infection nor a standardized serology for cats. In addition, some cats

with *E canis* infection do not seroconvert, and so PCR assay is superior to serologic tests in cats. PCR assays can be designed to amplify the nucleic acid in each organism. Alternately, primers are available to amplify the entire nucleic acid of the organisms in a single reaction, and then sequencing can be used to determine the infective species.

Cats can be infected by *Rickettsia felis* and have been shown to have antibodies against *Rickettsia rickettsii*. Fever, headache, myalgia, and macular rash in humans have been attributed to *R felis* infection in several countries around the world. In a study, 92 pairs of cat blood and flea extracts from Alabama, Maryland, and Texas were assayed using PCR assays that amplify a region of the citrate synthase gene (*gltA*) and the outer membrane protein B gene (*ompB*). Of the 92 pairs, 62 (67.4%) flea extracts and none of the cat blood samples were positive for the presence of *R felis* DNA.[26] In another study, antibody prevalence rates of *R felis* and *R rickettsii* were shown to be 5.6% and 6.6%, respectively, in cats with fever, but neither DNA was amplified from blood.[27] These results proved that cats are sometimes exposed to these organisms, but further data are needed to determine the significance of disease associations. Whether *Rickettsia* spp PCR assays are indicated for use in cats at present is unknown.

Blood culture, PCR assay on blood, and serologic testing can be used to assess individual cats for *Bartonella* spp infection. Cats that are culture negative or PCR negative and antibody negative and cats that are culture negative or PCR negative and antibody positive are probably not a source of flea, cat, or human infection. However, bacteremia can be intermittent and false-negative culture or PCR results can occur, limiting the predictive value of a single battery of tests. Although serologic testing can be used to determine whether an individual cat has been exposed, both seropositive and seronegative cats can be bacteremic, limiting the diagnostic utility of serologic testing. Thus, testing healthy cats for *Bartonella* spp infection is not recommended at present.[28] Testing should be reserved for cats with suspected clinical bartonellosis. Because *Bartonella* spp infection is so common in healthy cats, even culture- or PCR-positive results do not prove clinical bartonellosis. For example, although DNA of *Bartonella* spp was detected in more number of cats with fever than in pair-matched cats without fever, the test results in healthy cats were still commonly positive.[29] A combination of serology and PCR is a rational approach to the evaluation of cats with suspected bartonellosis.

Cytauxzoon felis in clinically affected cats is usually easily identified on cytologic examination of blood smears or splenic aspirates. Serologic testing is not commercially available. PCR can be used to amplify the organism's DNA from the blood of cats that are cytologically negative for *Cytauxzoon felis*.[30]

Antibodies against FIV are detected in serum in clinical practice most frequently by enzyme-linked immunosorbent assay (ELISA). Comparisons between different tests have shown that the results of most assays are comparable.[31] Results of virus isolation or RT-PCR on blood are positive in some serologically negative cats. False-positive reactions can occur using ELISA; hence, positive results of ELISA in healthy or low-risk cats should be confirmed using Western blot immunoassay. Kittens can have detectable colostrum-derived antibodies for several months. Kittens younger than 6 months that are FIV seropositive should be tested every 60 days until the result is negative. If antibodies persist at 6 months of age, the kitten is likely infected. Virus isolation or RT-PCR on blood can also be performed to confirm infection. However, FIV is not present in the blood in high levels, and so false-negative test results are common. Thus, the assay is not very accurate for distinguishing a vaccinated cat from a naturally exposed cat.[32,33]

Most cats with feline leukemia virus infection are viremic, and so molecular diagnostic assays are not usually needed in clinical practice. However, newer sensitive qPCR assays have been used to accurately characterize the stages of infection[34,35] but these assays are not commonly available commercially.

RNA of both FIP virus and feline enteric coronavirus (FECV) can be amplified from the blood of cats, and so positive test results do not always correlate with the development of FIP. Amplification of the mRNA (messenger RNA) of the M gene by RT-PCR had mixed results in 2 studies performed to date. This amplification is a logical approach in theory and was found to have high specificity in the first report of this approach.[36] However, in a follow-up study with a larger number of cats, 13 of 26 apparently normal cats were positive for FECV mRNA in blood suggesting that the PPV of this assay for the diagnosis of FIP was low.[37] This assay is still available commercially; however, based on the published data, the assay does not seem to be anymore clinically useful than any other molecular assay for the diagnosis of FIP.

Ocular Agents

T gondii, *Bartonella* spp, FHV-1, and coronavirus are the organisms in which the DNA or RNA has been amplified most frequently from the aqueous humor of cats with endogenous uveitis. Although little is known about the predictive value of these assays when used with aqueous humor, the combination of molecular assays with local antibody production indices may aid in the diagnosis of some cases.

SUMMARY

As molecular tools become more widely available, the cost and availability of molecular assays become more accessible to feline practitioners. However, molecular diagnosis is a rapidly expanding field, and the sensitivity of these assays along with the often high frequency of detection in healthy animals makes interpretation of positive test results difficult. The clinician must remember that predictive value is a much more valuable tool for the assessment of the utility of a test result in a particular animal.

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