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CHAPTER 32

PCR in Infectious Disease Diagnosis and Management

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The ready availability of a correct etiologic diagnosis, particularly in the setting of contagious infections, enables the veterinarian to make early decisions regarding patient care and management, address appropriate treatment, and effect timely notification and discussion of management issues pertaining to the prevention of disease spread. The past two decades have seen a revolution in the understanding, management, diagnosis, control, and prevention of infectious diseases. This period has encompassed the discovery of emerging equine agents, antimicrobials, and vaccines as well as a wealth of improved diagnostic tests and molecular testing methods for equine practitioners. Despite these advances, infectious diseases remain a leading cause of equine morbidity and mortality, with resurgence of certain infections, an increasing population of elderly and more susceptible horses, and an increasing level of international equine commerce expanding the geographic distribution of pathogens. The focus of rapid diagnosis of infectious diseases also has shifted during this time. The most obvious change has been the appearance and increasing importance of nucleic acid (NA) amplification–based techniques, primarily polymerase chain reaction (PCR), at the expense of traditional methods of clinical microbiology. Polymerase chain reaction has become an increasingly important tool in microbial diagnosis in recent years because of its rapidity, affordability, high sensitivity, and high specificity. These characteristics have propelled the field of PCR-based molecular diagnostics into the arena of applied diagnostics for infectious agents. Because the number of published and offered PCR assays is steadily rising, there is a need for critical evaluation, comparison of performance, and eventually also standardization of methods to enable equine practitioners to select the optimal methodology.

Key features for the adoption of molecular diagnostics for infectious agents are (1) superior sensitivity and specificity compared with most immunoassays; (2) automated platforms that significantly increase throughput; (3) quantitative assessment of pathogen load, which is clinically useful; (4) fast turnaround time that speeds detection and reduces overall costs; and (5) simultaneous analysis of multiple analytes.

MOLECULAR AWARENESS AND TESTING STRATEGIES

Many veterinarians are aware of the availability of molecular diagnostic tests and have used these techniques in their practice. However, the lack of a market dominator for molecular diagnostics and the relatively fragmented market leads to confusion. This confusion is mostly based on a lack of directed education within the veterinary community. Most veterinarians rely on continuing education offered at local or national meetings to improve their knowledge base of molecular diagnostics. As more and more practitioners use PCR to diagnose infectious diseases, an understanding of the involved processes is important. Further, the indications for using PCR and interpretation of results are often confusing and warrant more education within the veterinary community. The differences among laboratories in protocols used add to the confusion caused by the lack of an acceptable standard.

Parallel testing for multiple infectious agents in highly standardized platforms is a central component of molecular assays; it essentially allows several detections, for both DNA and RNA pathogen targets, to happen simultaneously on a single sample. This development is a noteworthy driver for molecular diagnostics because it allows acquisition of more meaningful data from a single sample. This so-called panel strategy enables efficient workup of complex clinical syndromes with general or nonspecific clinical signs. These clinical situations do not allow for easy diagnostic decision making by the veterinarian because multiple infectious agents can be responsible for a given clinical picture. Even though veterinarians tend to make a single-pathogen diagnosis, it has become more evident in recent years that many syndromes are caused by coinfections. Panel testing on a large scale will uncover unknown dual or triple infections in animals, which can diffuse the clinical picture. It has long been speculated that seemingly clinically irrelevant equine herpesvirus type 2 (EHV-2) infections in horses may actually aggravate and diffuse the clinical picture presented by secondary infections. More characteristic examples are known from companion animal respiratory infections, which are often initiated by a subclinical virus infection that leads the way to secondary infections.

PREANALYTICAL VARIABLES AND RESULT INTERPRETATION

In general, molecular diagnostic laboratories provide precise recommendations for sample collection and shipping. These instructions pertain to specimen type, volume, anticoagulant, transport specifications, storage, and handling. The sample type or types needed are largely influenced by the pathogenesis of the disease and play a key role in the performance and interpretation of the test results. Veterinarians are advised to adhere to these recommendations because the quality of the result is directly correlated to quality of the sample and preservation of the nucleic acid content. Whole blood samples are collected aseptically into evacuated blood tubes containing EDTA; body fluids (e.g., thoracic, abdominal, joint, cerebrospinal, tracheal wash, bronchoalveolar, and guttural pouch lavage fluid) and tissues should be collected into serum tubes without additives; nasal or nasopharyngeal secretions should be collected with rayon- or Dacron-tipped swabs and are best kept in a serum or conical tube; fecal material should be collected into small fecal cups or serum tubes. All samples must be sent cooled on blue ice by express

mail overnight to the laboratory. Freezing of samples should be avoided because of the detrimental effects of the thawing process on NA. Short-term storage for a period of 2 to 3 days before shipment (such as would be necessary over a weekend) should be done in a refrigerated compartment. Each sample should be properly labeled and accompanied by a submission form containing information on the animal, owner, veterinarian, sample, and suspected pathogens. Most submission forms can be downloaded from the respective laboratory's website. The laboratory should be notified in advance, and inquiry should be made about the availability of the offered tests as well as the expected turnaround time and the associated costs. Incoming samples normally are processed the same day, and PCR results usually are available within 24 to 72 hours (including shipping) if the purified NA passes the internal sample quality controls (confirming proper collection, storage, shipping, and NA extraction) and other associated quality controls, such as PCR-positive and PCR-negative controls, internal positive control (to confirm absence of PCR inhibitors), and negative extraction control (to confirm absence of cross-contamination during the NA extraction process). Veterinarians should be aware of the quality controls run on their diagnostic samples by inquiring with the respective diagnostic laboratory.

Interpretation of results obtained with molecular assays for infectious diseases necessitates understanding of the pathogenesis and biology of the target organisms. Some challenges are unique to molecular tests and are different from considerations in interpreting other microbiologic tests. Such differences are related to the distinction between viable and nonviable organisms and the correlation of NA detection with presence of disease or disease association.

Interpretation of a negative result requires taking into consideration information about the sensitivity of the PCR test, limit of detection, and the NA extraction efficiency as indicated by the use of quantitative internal sample controls. A false-negative result may be caused by a degraded or unstable sample. Insufficient or inappropriate sample type, inadequate sampling procedures, and transport problems are additional sources of false-negative results. Sample-specific internal positive sample controls targeting endogenous genes, such as the universal 18S rRNA (single-stranded rRNA) or the glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene, help to overcome this problem, particularly if the lab uses them in a quantitative fashion to assess the quantity of NA going into a PCR protocol, which directly influences the limit of detection of the assay. In addition, inhibition phenomena originating from sample matrixes, such as feces, urine, or environmental samples contaminated with soil or surface water, have to be controlled with internal positive controls to assess the inhibitory effects on the PCR process.

The factors to consider for interpretation of positive results include assay specificity and contamination issues. Polymerase chain reaction or any other target amplification method is subjected to these considerations. Real-time PCR with closed-tube detection procedures reduces the risk for PCR product carryover as a source of false-positive results.

In general, molecular assays do not provide information about the viability of an infectious agent. Exceptions to this are DNA viruses, bacteria, and parasites that are analyzed for the presence of RNA molecules, such as rRNA and transcribed genes in the form of messenger RNA, instead of their genomic DNA equivalents. Targeting spliced RNA occurring at certain steps during the replication cycle of particular viruses provides additional information into the replication activity of a virus. In other cases, targeting the ribosomal RNA of parasites such as *Toxoplasma* spp and *Cryptosporidium* spp is a means of obtaining viability information and also may increase the analytical sensitivity.

Detection of a pathogen's NA in a sample does not necessarily indicate that the organism is the cause of the disease. However, using the quantitative information of a real-time PCR result may give further insight and provide a means for evaluating disease association. Primary examples are herpesvirus infections (EHV-1 and EHV-4), in which the quantitative detection of DNA may indicate presence of lytic, nonreplicating, or latent virus. Studies have indicated that high viral loads of EHV-4 and EHV-1 DNA allow formulation of laboratory-specific cutoff values to differentiate between lytic and nonreplicating virus. In such cases, high viral loads are generally associated with the presence of clinical signs and the presence of viral RNA transcripts indicating virus replication. Therefore quantitative real-time PCR can provide a means of obtaining information about the disease association, a crucial criterion for the equine practitioner in making the correct diagnosis.

Veterinarians can use a variety of guidelines to select laboratories for molecular diagnostic testing. Certain questions are worth asking before samples are submitted to a molecular diagnostic laboratory. These questions should cover three areas. First, it is worthwhile to obtain information about the nature of the PCR testing platform (traditional versus real-time). Second, questions should be asked about the quality control and quality assurance system within a particular laboratory. In particular, it is useful to know whether whole processes are controlled or just single point controls are used, and how contamination is avoided and confirmed to be absent within the laboratory. Third, additional questions about turnaround time, pricing, and the level of guidance with result interpretation are worth asking before samples are submitted.

DETECTION OF COMMON EQUINE PATHOGENS

To facilitate a decision about which pathogens should be evaluated for a specific case, many modern molecular laboratories offer panels covering specific organ systems (e.g., respiratory, gastrointestinal, or nervous system). Such panels test several common pathogens for each organ system. The diagnostic PCR applications most relevant for equine practice are summarized (Table 32-1).

Respiratory Pathogens

Despite intensive investigative efforts, veterinarians frequently diagnose clinical infectious respiratory tract diseases without identifying a primary etiologic agent. A recent voluntary surveillance study on 761 equids in the United States with clinical signs of acute-onset respiratory tract infection determined that 26.4% of index cases had positive PCR results for one or more of four selected common respiratory pathogens (EHV-1, EHV-4, equine influenza virus [EIV], and *Streptococcus equi* subsp *equi*). The highest detection rate was for EHV-4, followed by EIV, *S equi equi*, and EHV-1. The absence of etiologic diagnosis for some infectious respiratory tract disease cases observed in this and other studies is at least partially attributable to concentrating diagnostic efforts on identifying infection with agents that most frequently cause disease. It is likely that more comprehensive diagnostic efforts would identify agents in affected animals that tend to cause either less dramatic outbreaks or sporadic rather than epidemic disease (e.g., γ-herpesviruses and equine rhinitis viruses).

TABLE 32-1 Diagnostic PCR Assays for Equine Pathogens With Biologic Sample Type Used for Detection

Pathogen	Biologic Sample Type Used for Detection
Anaplasma phagocytophilum	Whole blood
Corynebacterium pseudotuberculosis	Aspirate from abscess, body fluid
Equine arteritis virus	NPS, TW, BAL
Equine coronavirus	Feces
Equine influenza virus	NPS, TW, BAL
Equine herpesvirus type 1	NPS, TW, BAL and whole blood
Equine herpesvirus type 4	NPS, TW, BAL
Equine rhinitis A and B virus	NPS
Equine rotavirus	Feces
Lawsonia intracellularis	Feces and blood for serology
Neorickettsia risticii	Feces and whole blood
Salmonella spp	Feces, selective enrichment broth
Streptococcus equi subsp *equi*	NPS, NPL, GPL, lymph node aspirate

BAL, Bronchoalveolar lavage fluid; *GPL,* guttural pouch lavage; *NPL,* nasopharyngeal lavage; *NPS,* nasal/nasopharyngeal swab; *TW,* tracheal wash fluid.

The sample of choice for the molecular detection of viruses associated with infectious respiratory tract disease is nasal secretions, which are generally collected from the nasal passages or nasopharynx by use of rayon- or Dacron-tipped swabs. The use of viral transport medium for the transportation of nasal swabs is not necessary for PCR detection because NA-based assays do not rely on viability of the target pathogen. Polymerase chain reaction assays testing for the presence of EIV, EHV-1, EHV-4, and *S equi equi* have superior sensitivity, compared with antigen-capture enzyme-linked immunosorbent assays (ELISAs) and conventional culture systems. Another advantage of molecular assays is their ability to detect nonviable virus, a situation that may occur when nasal or nasopharyngeal samples are frozen or not adequately stored or shipped to a diagnostic laboratory. Further, novel PCR platforms do allow quantitation of DNA or RNA content in a given sample. This is of interest to assess the kinetics of viral shedding, to determine the infectious nature of a clinically or subclinically infected horse, and to assess response to treatment.

Equine influenza virus is routinely detected from nasal secretions collected from horses during the early febrile stage of the disease (see also Chapter 39). Amplification of the single-stranded RNA of EIV is performed by reverse transcriptase PCR (RT-PCR) technology, using either a one-step, nested, or real-time approach. The hemagglutinin, nucleoprotein, and matrix genes are the commonly targeted genes for these molecular assays. Nucleotide and deduced amino acid sequences of portions of the hemagglutinin gene are now routinely used for phylogenetic characterization of outbreak strains.

Equine herpesvirus types 1 and 4 are double-stranded DNA α-herpesviruses that infect the equine respiratory tract and can establish lifelong latent infection after exposure (see also Chapter 37). The diagnostic sample of choice is a nasal or nasopharyngeal swab, which should be collected early in the febrile phase of the disease. Because of the lymphotropism of EHV-1, detection can also be attempted from whole blood. The PCR assays used in the diagnostic field are based on detection of viral genomic DNA and are therefore unable to distinguish between lytic, dead, or latent virus. Alternative molecular approaches have recently been established using quantitative real-time PCR to allow discrimination between the different viral states in horses naturally infected with EHV. Discrimination between the different viral states is now possible by (1) targeting several genes (e.g., glycoprotein, latency-associated transcripts), (2) detecting viral genomic DNA and transcriptional activity of the target genes at the messenger RNA level, and (3) using absolute virus quantification. Quantitative thresholds are used in selected human infectious diseases (e.g., human immunodeficiency virus, hepatitis C virus, and herpes simplex virus) to determine disease stage and response to antiviral therapy. A similar concept is used diagnostically for EHV-1– or EHV-4–infected horses to discriminate between lytic and nonreplicating viruses, to determine their infectious risk based on viral load in nasal secretions, and to monitor their response to treatment.

Streptococcus equi subsp *equi* infection rarely is associated with detection difficulties when conventional culture is used in clinically affected horses (see also Chapter 41). Culture of nasal swabs, nasopharyngeal or guttural pouch washes, or exudate aspirated from an abscess remains the gold standard for detection of *S equi equi*. Culture, however, may be unsuccessful during the incubation and early clinical phase of infection. Further, the presence of other β-hemolytic streptococci, especially *S equi* subsp *zooepidemicus*, may complicate interpretation of the culture. Available PCR assays are designed to detect the DNA sequence of the *S equi* M protein *(SeM)* gene, which codes for the organism's antiphagocytic M protein. This gene offers sufficient nucleotide variability between the two *S equi* subspecies to allow full discrimination in clinical specimens. One of the pitfalls of PCR has been its inability to distinguish between viable and nonviable organisms; therefore positive results have been considered presumptive in the past until confirmed by culture. At present, the question of viability can be addressed by quantitation of the *SeM* gene or detection of transcriptional activity of the *SeM* gene at the RNA level. In several studies, PCR proved to be as much as three times as sensitive as culture. Use of PCR accompanying culture on a nasal swab or guttural pouch lavage sample may be advantageous in a control program to select possible carrier animals because PCR is capable of detecting *S equi* DNA in guttural pouch fluid for weeks after the disappearance of live organisms. Such is not the case for the nasopharynx, in which the efficient mucociliary apparatus removes organisms and DNA at the same time. Use of PCR should be considered to detect asymptomatic carriers, to establish the *S equi* infection status of asymptomatic horses, and to determine the success of elimination of *S equi* from the guttural pouch. Unfortunately, diagnostic PCR assays are unable to differentiate between wild-type and the nonencapsulated, avirulent vaccine strains of *S equi*.[1] However, when necessary for forensic reasons, differentiation can be undertaken on the basis of morphology of colonies, biochemical analysis, genotyping, and restriction digest. Together, these assays allow differentiation between wild-type and vaccine or ancestor strains.

[1]Pinnacle I.N., Zoetis, Kalamazoo, MI.

Equine rhinitis A and B virus (see also Chapter 38) and *equine arteritis virus*, although less commonly associated with infectious upper respiratory tract disease, should also be considered as target pathogens during respiratory outbreaks. The role of EHV-2 and EHV-5 in nasal secretions of horses with infectious respiratory disease is still unclear. Given their high prevalence in horse populations, and in order to avoid dilemmas with the interpretation of PCR results, testing for γ-herpesviruses is not recommended at present.

Neurologic Pathogens

Although they are highly sensitive and specific, PCR assays have not been developed for the detection of viral and protozoal pathogens in the cerebrospinal fluid (CSF) of neurologic horses. These methods often are of limited value in routine diagnosis because either the viremia is very short lived or the pathogen has no affinity for the nucleated cells in CSF. Consequently, pathogens are usually no longer detectable at the onset of systemic or neurologic signs. One exception is the uncommon neurologic form of EHV-1 infection, known as *equine herpesvirus myeloencephalopathy* (EHM).

A diagnosis of EHM is supported by historical and clinical findings (see also Chapter 36), the presence of xanthochromia and high total protein concentration in CSF, and laboratory detection of EHV-1 in blood or nasal secretions by PCR. Because affected horses can shed the virus in nasal secretions and thus represent a risk for infection for unaffected in-contact horses, it is imperative to determine the risk for shedding in a suspect horse to initiate an appropriate infectious disease control protocol. The dilemma as to whether the virus is in a lytic, nonreplicating, or latent state can be addressed by use of absolute quantitation or transcriptional activity of the target gene, similar to the approach used for EHV-4. Research groups have recently identified regions of variation in the genome of different EHV-1 strains (neuropathogenic vs. nonneuropathogenic). A single nucleotide polymorphism at position 2254 of the DNA polymerase gene (ORF 30) has been associated with a higher risk for EHM development. Rapid PCR assays have been established to allow differentiation between neuropathogenic and nonneuropathogenic strains. However, such assays have moderate specificity because 74% to 87% of EHV-1 strains associated with EHM are of the neuropathogenic genotype. Therefore these assays should be used judiciously, and the results should always be interpreted in the context of clinical presentation. Further, these assays should be coupled with additional assays targeting conserved regions of the EHV-1 genome.

Gastrointestinal Pathogens

The detection of equine gastrointestinal pathogens with conventional or molecular tests can be challenging because these pathogens either are difficult to grow in cell culture systems or can be present in pathogenic or nonpathogenic forms, making interpretation of positive results difficult. Furthermore, the use of fecal material for molecular diagnostics has been associated with false-negative results because of inhibitory substances in the feces that can interfere with NA extraction or amplification. However, development and use of specific extraction kits and derivation of a set of appropriate controls (internal positive control) have improved the yield and quality of NA from feces and expanded the usability of molecular methods. As with other biologic sample types, it is important that sample quality and inhibition be monitored with internal or external controls.

Neorickettsia risticii, agent of Potomac horse fever (PHF), causes a serious enterocolitis in horses of all ages. The diagnosis of PHF is based on the detection of *N risticii* from blood or feces of infected horses. Isolation of the agent in cell culture, although possible, is time consuming and not routinely available in many diagnostic laboratories. The development of *N risticii*–specific PCR assays has greatly facilitated diagnosis of PHF. These molecular assays have been key in the investigation of the epidemiology of PHF, allowing the discovery of helminthic vectors and intermediate and definitive helminthic hosts as well as determining the natural route of infection. Although NA of *N risticii* can be detected in the blood and feces of naturally or experimentally infected horses, the detection period does not necessarily coincide between the two sample types. It is therefore recommended to analyze both types of biologic samples from horses suspected of having PHF to enhance the chance of molecular detection of *N risticii*.

Lawsonia intracellularis, agent of equine proliferative enteropathy (EPE), is an emerging equine gastrointestinal pathogen of young horses (see also Chapter 79). Because culture of *L intracellularis* from feces is not possible at present, antemortem diagnosis relies on serology and PCR. The combination of both tests increases the chance of diagnosing EPE. Testing with PCR has the advantage of being fast and able to yield positive results in the early stage of disease, when antibodies are not yet measurable. Prior use of antimicrobials can negatively affect the molecular detection of *L intracellularis* in feces. Therefore, in a suspected case, fecal collection for PCR testing should be performed before initiating any antimicrobial treatment.

In recent years, PCR assays for the detection of *Salmonella* spp in fecal samples from horses admitted to veterinary hospitals have been evaluated. Collectively, these studies have unquestionably reported higher analytical sensitivity for the detection of *Salmonella* spp through PCR assays compared with conventional microbiologic culture. The higher detection rate of *Salmonella* by PCR has been attributed to the detection of nonviable organisms and of previously undescribed *Salmonella*-like bacterial organisms. The use of novel virulence target genes for the molecular detection of *Salmonella* has considerably improved the performance and accuracy of such assays. More and more veterinary hospitals in North America are switching from conventional microbiologic culture to PCR for *Salmonella* testing as part of the infectious disease control program. In such instances, PCR is performed on fecal and environmental samples following a 24-hour selective enrichment step. The use of PCR is very cost effective and has the potential to reduce contamination risks and turnaround time, with results available 22 to 28 hours from sample collection (e.g., 18 to 24 hours enrichment time plus 4 hours for DNA purification and amplification). Further, the use of absolute quantitation allows assessment of the infectious nature of hospitalized animals and may be an excellent alternative to conventional culture methods for surveillance and research studies.

The detection of *equine coronavirus* (ECoV) by PCR in the feces of foals with fever and diarrhea is difficult to interpret because ECoV has also been detected in the feces of healthy foals. Healthy foals have been found to be infected by ECoV as a single infection, without other coinfecting agents, whereas ECoV in sick foals has been found exclusively in association with other coinfecting agents. This finding is in agreement with the behavior of coronaviruses in other species, in which the virus may not have enough pathogenic potential to cause disease, but causes local immune

suppression and enables secondary infections to become established more efficiently. In adult horses, ECoV causes self-limiting disease characterized by depression, inappetence, fever, and, less frequently, changes in fecal character and colic. More epidemiologic studies are needed to better understand the impact of this emerging disease.

Equine rotavirus poses a challenge each foaling season to farm managers and veterinarians in intensive horse breeding areas throughout the world. A quick and reliable diagnosis is essential to separate affected foal with diarrhea and reduce the spread of this virus. Diagnosis of rotavirus infection in the past has relied on direct virus detection with a rapid antigen-capture ELISA. Recently developed PCR assays have high analytical sensitivity, specificity, and accuracy in the diagnosis of equine rotavirus infection and will likely replace the less sensitive ELISA test in the near future.

Miscellaneous Pathogens

Equine granulocytic anaplasmosis is caused by *Anaplasma phagocytophilum*, a rickettsial pathogen transmitted by *Ixodes* spp ticks. Diagnosis is often based on awareness of the geographic area for infection, typical clinical signs, abnormal laboratory findings, and identifying characteristic pathogen inclusions in the cytoplasm of neutrophils and eosinophils in a peripheral blood smear. Polymerase chain reaction has been used for many years to study aspects of the epidemiology and pathophysiology of equine granulocytic anaplasmosis. For clinical purposes, the material of choice is whole blood; PCR is a very sensitive and specific tool, supporting the diagnosis especially during the early and late stages of the disease, when the number of organisms is too small to be detected by microscopy.

Corynebacterium pseudotuberculosis is a common cause of external and internal abscesses in horses from arid regions of North America. The epidemiology has recently been investigated with the help of PCR, and flies have been identified as mechanical vectors. *C pseudotuberculosis* is easy to grow in culture, and the indications for PCR are restricted to specific situations (e.g., when aspirates or body fluids are culture negative).

Additional PCR assays for *Borrelia burgdorferi sensu lato, Leptospira* spp, *Mycobacterium* spp, *Mycoplasma* spp, *Babesia caballi, Theileria equi, Clostridium difficile* (antigen and toxin A and B), toxigenic *Clostridium perfringens, Cryptosporidium* spp, and methicillin-resistant *Staphylococcus aureus* have been developed and are being used in the research setting. These assays will likely be offered in the near future for diagnostic purposes when additional epidemiologic information and accuracy of the tests have been validated on clinical samples from infected horses.

Suggested Readings

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