Review

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Real-Time Polymerase Chain Reaction: A Novel Molecular Diagnostic Tool for Equine Infectious Diseases

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The focus of rapid diagnosis of infectious disease of horses in the last decade has shifted from the conventional laboratory techniques of antigen detection, microscopy, and culture to molecular diagnosis of infectious agents. Equine practitioners must be able to interpret the use, limitations, and results of molecular diagnostic techniques, as they are increasingly integrated into routine microbiology laboratory protocols. Polymerase chain reaction (PCR) is the best-known and most successfully implemented diagnostic molecular technology to date. It can detect slow-growing, difficult-to-cultivate, or uncultivatable microorganisms and can be used in situations in which clinical microbiology diagnostic procedures are inadequate, time-consuming, difficult, expensive, or hazardous to laboratory staff. Inherent technical limitations of PCR are present, but they are reduced in laboratories that use standardized protocols, conduct rigid validation protocols, and adhere to appropriate quality-control procedures. Improvements in PCR, especially probe-based real-time PCR, have broadened its diagnostic capabilities in clinical infectious diseases to complement and even surpass traditional methods in some situations. Furthermore, real-time PCR is capable of quantitation, allowing discrimination of clinically relevant infections characterized by pathogen replication and high pathogen loads from chronic latent infections. Automation of all components of PCR is now possible, which will decrease the risk of generating false-positive results due to contamination. The novel real-time PCR strategy and clinical applications in equine infectious diseases will be the subject of this review.

Key words: Horse; Pathogen detection; TaqMan polymerase chain reaction.

Equine practitioners have always considered accurate and rapid identification of infectious pathogens a priority. The ready availability of a correct etiologic diagnosis, particularly in contagious infections, enables the veterinarian to make early decisions on the patient's care and management, address appropriate treatment, and allow timely notification and discussion of management issues regarding prevention of disease spread. The last 2 decades have seen a revolution in the understanding, management, diagnosis, control, and prevention of infectious diseases.[1,2] This period has encompassed the discovery of emerging equine agents, antimicrobials, vaccines, as well as a wealth of improved diagnostic tests for equine practitioners. Despite these advances, infectious diseases remain a leading cause of equine morbidity and mortality, with resurgence of certain infections (eg, West Nile virus), an increasing population of elderly, more susceptible horses, and an increasing international equine commerce, expanding the geographic distribution of pathogens.[3,4]

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 the polymerase chain reaction (PCR), at the expense of traditional methods of clinical microbiology.[5] The introduction of NA detection methods into routine diagnostics represents an important step forward. A 2nd-generation PCR method called real-time PCR uses a probe-based detection system allowing PCR amplification and detection within a closed-tube system.[6,7] Real-time PCR technology can be used as an endpoint (qualitative) PCR or as quantitative PCR. Closed-tube detection of PCR product accumulation and therefore elimination of post-PCR handling steps provides a system without risk of PCR product carryover, resulting in a decreased likelihood of false-positive results.[8] Still, implementation of NA technology into routine diagnostics has raised new and confounding questions concerning its use to diagnose clinical infectious diseases.

This review intends to demystify PCR diagnostic testing for the equine practitioner. Here, we offer insight into laboratory standards using a 2nd-generation PCR platform called real-time PCR. Present and future clinical applications in equine infectious diseases also are reviewed.

Sample Submission

NA techniques to detect the presence of infectious agents in biological specimens require stringent quality guidelines. These guidelines aim to assure the stability of NAs (both genomic DNA and total RNA), which are the target for molecular-based diagnostic methods. Whole-blood samples are collected aseptically into evacuated blood tubes containing ethylenediaminetetraacetic acid (EDTA)[a]; body fluids (eg, thoracic, abdominal, joint, cerebrospinal, transtracheal wash, bronchoalveolar, and guttural pouch lavage fluid), swabs, and tissues should be collected into serum tubes without additives; fecal material should be collected into small fecal cups or serum tubes. All samples must to be sent cooled on blue ice by express mail overnight to the laboratory. Freezing of samples should be avoided. Short-term storage for a period of 2–3 days before shipment (over a weekend) should be done in a refrigerated compartment. Each sample should be accompanied by a submission form containing information on the animal, owner, veterinarian,

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sample, and pathogen(s) to be tested (most submission forms can be downloaded from the respective laboratory website). The laboratory should be notified in advance and inquiry should be made about the availability of the offered tests as well as turn-around time and associated costs. Incoming samples normally are processed the same day and PCR results usually are available within 24–48 hours if the NA passes quality control.

Nucleic Acid Preparation

Molecular-based detection methods including PCR require the release of NA from the sample material. Endogenous enzymes able to digest DNA and RNA (DNases and RNases) are neutralized by using lysis reagents that can disrupt cell walls and denature proteins. To allow standardized NA extraction, commercially available NA extraction kits[b] or automated NA workstations[c] facilitate collection of high-quality material. To detect RNA viruses, viral and cellular RNA is transcribed into complementary DNA (cDNA) by an enzymatic process called reverse transcription (RT-step). This process can be performed manually or using a NA workstation.[c]

Nucleic Acid Quality Controls

The DNA and RNA used for PCR amplification must pass rigid quality control to increase the reliability of a negative PCR result. Because cellular NA is coextracted with NA from the pathogen, cellular NA can be targeted by a separate PCR reaction, allowing a quality assessment of the NA. In addition, real-time PCR provides quantitative results and, therefore, the quality control obtained on the cellular NA provides quantitative information about the amount of NA present in each assay. By comparing the quantitative quality-control signals from clinical samples with laboratory standards, a threshold can be defined for each sample type. Negative PCR results for a pathogen from samples not passing quality control must be interpreted with caution and should be repeated on a back-up sample or new clinical specimen.

PCR Amplification Method

The Conventional Approach

PCR is an enzymatic procedure that allows almost unlimited amplification of specific NA sequences in vitro. During the process, the double-stranded target DNA is multiplied in repetitive cycles (Fig 1). The elements of any PCR are 2 well-defined primers that bind specifically to 1 of each of the 2 DNA strands, sufficient amounts of deoxynucleotides, a heat-stable DNA polymerase, and a buffer of well-defined composition. The sequences of the 2 primers must be carefully selected for perfect match with the target DNA. Primers with a typical length of 25–30 base pairs are synthesized.

Typical volumes of a PCR assay are 10–50 μL. The reaction is carried out in PCR tubes specifically designed to fit tightly into cavities of an aluminum block, which can be heated and cooled in alternate cycles. The process usually is stopped after 35–40 cycles and the product visualized by gel electrophoresis and subsequent staining. During gel electrophoresis, the amplified DNA is separated according to the size of the molecule (ie, small DNA pieces migrate far and larger pieces only a small distance from the point of application). After gel electrophoresis, the gel is soaked in a buffer containing a dye that specifically stains DNA, usually ethidium bromide, which shows intense fluorescence when illuminated by ultraviolet light.

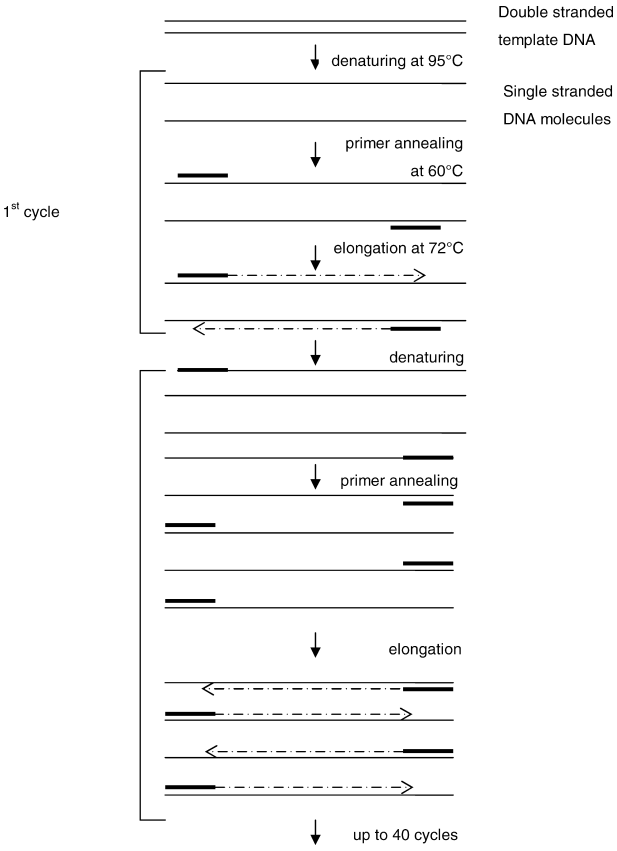

Fig 1. Principle of conventional polymerase chain reaction (PCR). A double DNA strand is denaturated by heat (95°C) resulting in two single strands (denaturation). The temperature is then lowered, for example to 60°C, which allows the primers (—) to bind to their complementary sequence (Primer annealing). The temperature is then increased to 72°C, the optimal temperature for *Taq* polymerase, which extends the primers at the 3′ end (elongation). After extension, 4 strands (2 double strands) will have resulted from the 2 original strands. In cycle 2, the same process is repeated, resulting in 8 strands and so on.

The Real-Time Approach

Technological advances in PCR thermocycler performance and fluorescent signal detection in the early 1990s allowed introduction of a 2nd-generation PCR method in 1996 employing the TaqMan chemistry.[d] This method uses a fluorescent dual-labeled probe (TaqMan probe) added to the PCR reaction mix. The 5′ exonuclease activity of the DNA polymerase displaces and digests the TaqMan probe upon primer extension. This causes the release of the quenched fluorescent signal, which is measured during each PCR cycle in a closed-tube detection system, hence the term real-time (Fig 2). A digital camera collects fluores-

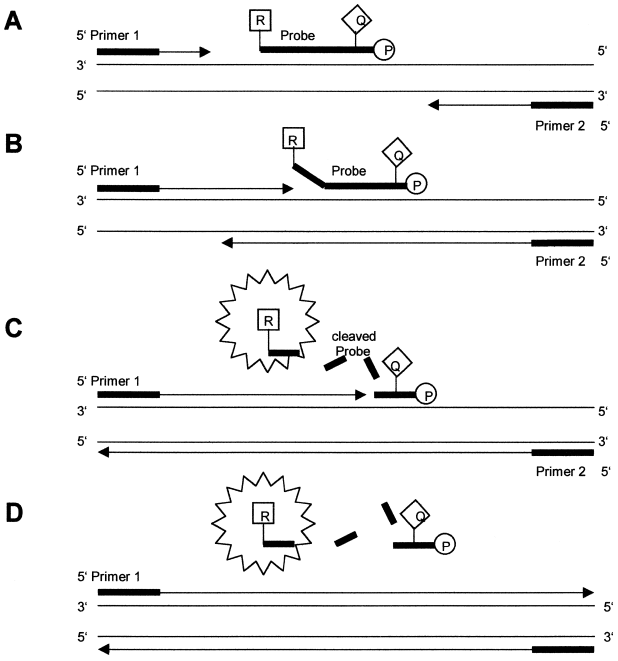

Fig 2. (**A**) Diagram of TaqMan 5′-3′ nuclease assay. Polymerase chain reaction (PCR) primers 1 and 2 and a TaqMan probe labeled with FAM reporter dye (R) and TAMRA quencher dye (Q) anneal to the DNA template. Extension of the TaqMan probe is blocked by the 3′ phosphate group (P). (**B**) *Taq* polymerase extends the primer and displaces the TaqMan probe. (**C**) The displaced probe is cleaved by *Taq* polymerase resulting in an increase in relative fluorescence of the reporter. (**D**) Polymerization completed.

cence signals in real-time and the data is stored on an attached computer; all post-PCR analysis is computer based and does not include any wet-lab components. Probe chemistries other than TaqMan (such as molecular beacons) can be used with identical performance characteristics. Real-time monitoring of PCR product accumulation leads to fully quantitative PCR systems that have replaced all conventional quantitative PCR methods for research applications. Different companies offer real-time PCR platforms that can run probe-based real-time PCR.

Conventional PCR protocols using gel electrophoresis for PCR product visualization still are widespread in veterinary diagnostic laboratories. These protocols carry an inherent risk of PCR product carryover and can lead to false-positive signal generation. A diagnostic laboratory can do little to prevent product carryover. Even the most rigorous separation of PCR mastermix preparation and post-PCR manipulation of products cannot eliminate the risk of PCR product carryover. Experience of the past 20 years has clearly showed that conventional PCR has important technical drawbacks for diagnostic application. With the rapid increase in accepted protocols for real-time PCR, conventional PCR eventually will become obsolete and give place for the more advanced real-time PCR platform. This change in the diagnostic arena is necessary for increased use and acceptance of PCR as a powerful diagnostic tool.

Taken together, probe-based real-time PCR offers important advantages over conventional PCR, including (1) quantitative quality control of the input target DNA; (2) elimination of postamplification steps, resulting in virtual absence of PCR product carryover and false-positive PCR product generation; (3) fast and efficient real-time PCR on 96- or 384-well plates leading to short turn-around time; (4) standardized PCR protocols with uniform amplification specifications leading to high reproducibility among diagnostic laboratories; and (5) availability of stable and quality-controlled PCR reagents contributing to high reproducibility and reliability.

Design and Validation of Probe-Based Real-Time PCR Protocols

Compared with conventional PCR protocols, real-time PCR provides increased analytical sensitivity and specificity. Probe-based real-time PCR assays can detect molecules in the single-copy range and can reproducibly detect 5–10 molecules. If this analytical sensitivity for any real-time TaqMan PCR system is not achieved, nucleotide mismatches or problems in generating the standard curve are possible reasons. Improved analytical specificity is achieved by the addition of a fluorescent-labeled probe to the assay. The probe will detect the specific PCR product only; any unspecific PCR products with a different sequence will not be detected.

Commercially available computer software packages[e] allow the design of a real-time PCR system including a TaqMan probe to conform to very rigorously defined standards.[9] All real-time PCR systems designed using computer-based software packages can be amplified with a standardized PCR cycling protocol, be used at uniform primer and probe concentrations, and be used with quality-controlled, commercially available PCR master mixes. Nucleotide sequence information has increasingly become available and facilitates the design of either highly specific assays for defined isolates or broadly specific assays for genus-wide detection of pathogens. As is the case for any PCR protocol, information on diagnostic sensitivity and specificity must be obtained for new protocols, but a large body of information is available in published protocols.

Advantages and Pitfalls of PCR as a Diagnostic Tool

PCR has distinct advantages as a diagnostic tool over conventional microbiology, especially in the detection of slow-growing, difficult-to-cultivate, or uncultivatable microorganisms. PCR is best used in clinical situations in which conventional clinical microbiology diagnostic procedures are inadequate, time-consuming, difficult, expensive, or hazardous to laboratory staff. PCR can be used successfully in situations in which inhibitory substances, such as antimicrobials, are present. Due to the stability of DNA, pathogen detection can be performed successfully on a variety of sample types or even using formalin-fixed tissue.

PCR also has inherent disadvantages. Real-time PCR addresses the PCR product carryover problem very efficiently. Additional sources of false-positive signal generation occur, however, such as inappropriate use of positive controls, the handling of plasmids (which may carry the target sequence of a PCR assay), and DNA contamination of samples dur-

ing the NA purification process. Diagnostic PCR laboratories should address these issues by using separate laboratories for reagent preparation, specimen processing, and amplification and detection, as well as by using automated and enclosed extraction systems and closed-tube PCR systems such as real-time PCR. Additional systems are available to prevent the reamplification of PCR products, such as the AMPErase UNG system.[f,10]

Diagnostic Applications of Real-Time PCR

Using the quantitative nature of real-time PCR, pathogen-load determination can help to answer pertinent questions related to the involvement of a pathogen in the disease process. As an example, quantitative HIV viral-load determination is the most important predictive parameter for disease outcome and is widely used for diagnostics, therapy monitoring, and therapy-resistance assessment.[11]

Viability Assessment Using PCR

Nucleic acid detection assays often are criticized as only detecting pathogens at the DNA level, which is not necessarily a reliable indicator of pathogen viability. Therefore, PCR-positive signals can be caused by environmental contamination in which presence of the pathogen is not associated with clinical disease. If viability assessment is of clinical importance, PCR analysis at the RNA level provides information about the transcription activity of the organism, which is a marker of viability. Such a procedure is not possible for certain RNA viruses but can be used for most bacteria, parasites, fungi, and DNA viruses.

Biomarker Definition for Disease Processes

Biomarkers are defined as genes that are specifically induced or downregulated during disease. These sets of genes, also called gene signatures, can identify a disease process with high confidence. Such biomarkers have been defined for a number of neoplastic diseases. Pathogens causing a disease process are able to induce host genes that are characteristic for a certain clinical entity. For example, cytokines or chemokines and their transcription factors and receptors are selectively induced and can be used to detect an infectious-disease process without the presence of the pathogen itself. Additionally, pathogen-specific genes encoding for antimicrobial resistance or toxins can be used as diagnostic biomarkers.

Single-Pathogen Detection Versus Panel Strategy

The selection of the PCR test to be used for the diagnostic procedure still relies on the ability of clinicians to indicate the suspected organism. In most cases, the clinician chooses a single PCR assay to be performed and relies on subjective assessment of available clinical and laboratory variables to identify the suspected pathogen. This hit-or-miss approach carries a risk of misdiagnosing the patient if a different pathogen is involved in the infectious disease process. By using a list (or panel) of pathogens able to induce a similar disease process, the diagnostic power of PCR can be greatly improved. Instead of selecting a single pathogen, the clinician selects a syndrome-specific panel of PCR assays that broadly covers potential pathogens.

Interpretation of Nucleic Acid Amplification Results

The majority of equine pathogens evaluated by laboratories are identified by qualitative endpoint PCR and lead to a yes-or-no answer. This approach is insufficient for pathogens such as equine herpesviruses, which are known to cause latent infection after primary exposure. The interpretation of a positive result for such latent pathogens requires a more specific strategy, such as absolute quantitation of viral DNA or PCR analysis at the RNA level, to allow direct correlation with clinical disease.[g] Contacting the laboratory and having a skilled person explain the results should be considered whenever results are confusing or conflicting.

Costs and Savings of Nuclei Acid Amplification Techniques

Although molecular diagnostic tests traditionally have been more expensive than conventional diagnostic techniques, the ease with which some molecular tests can now be performed and the rapid results generated by these methods can provide timely diagnosis and translate into overall savings. For example, a rapid PCR test method may replace labor-intensive cell culture methods previously used to detect viruses. Cost savings may be realized because rapid diagnosis may prevent other diagnostic procedures, limit unnecessary empirical antimicrobial therapy, and shorten hospital stays in expensive isolation facilities. Earlier detection of infectious agents also may limit the spread of contagious pathogens to healthy horses and increases the overall quality of the veterinary service.

Clinical Applications in Equine Infectious Diseases

An array of NA amplification techniques may be offered by laboratories in order to establish an etiologic diagnosis of equine infectious diseases. Several published studies have shown the benefit of NA amplification techniques in comparison with conventional microbiological techniques. Current efforts are aimed at improvement of the diagnostic efficiency of molecular techniques, both for frequent and less common infections. Table 1 presents an overview of real-time PCR assays routinely used for the detection of equine pathogens. To facilitate a decision on which pathogens should be evaluated, some laboratories offer panels covering specific organ systems (eg, respiratory, gastrointestinal, neurology). Such panels test several common pathogens for each organ system. Some of the diagnostic applications in real-time PCR most relevant for equine practice are presented below along with their advantages and potential pitfalls.

Respiratory Pathogens

Respiratory pathogens often are contagious, and infections must be diagnosed rapidly in order to prevent a disease outbreak and institute the appropriate management plan. The short turn-around time and reliability of real-time

Table 1. Examples of real-time TaqMan polymerase chain reaction (PCR) assays for the diagnosis of equine infectious diseases.

Microbial Pathogen	Tissue Submission	Target Gene	Reference
Anaplasma phagocytophila	Whole blood	16S rRNA	51
Corynebacterium pseudotuberculosis	Aspirate from abscess	Phospholipase D exotoxin	55
Equine herpesvirus 1	NPS, whole blood, TTW, BAL, CSF	Glycoprotein B	g
Equine herpesvirus 4	NPS, whole blood, TTW, BAL	Glycoprotein B, LATs	i
Equine influenza virus	NPS, TTW, BAL	Hemagglutinin	LWMDCF
Lawsonia intracellularis	Feces	16S rRNA	49
Neorickettsia risticii	Whole blood, feces	16S rRNA	39
Neospora hughesi	CSF	18S rRNA, ITS	i
Rhodococcus equi	TTW, BAL, feces	vapA	LWMDCF
Sarcocystis neurona	CSF	18S rRNA, ITS	i
Streptococcus equi	NPS, NPL, GPL, lymph node aspirate	M protein	LWMDCF
West Nile virus	Whole blood, CSF	Envelop gene	31

NPS, nasopharyngeal swab; TTW, transtracheal wash; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; NPL, nasopharyngeal lavage; GPL, guttural pouch lavage; LATs, latency-associated transcript; ITS, internal transcriber spacer; vapA, virulence-associated plasmid A; LWMDCF, assay established and validated by C.M. Leutenegger at the Lucy Whittier Molecular and Diagnostic Core Facility, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA.

PCR makes this molecular technology an ideal tool for the diagnosis of respiratory pathogens.

Equine influenza is commonly diagnosed by virus detection using an immunoassay[h] because of its rapid turnaround time. The sample of choice is a nasopharyngeal swab collected from a horse during the early stage of the disease. In a recent study comparing the sensitivity of virus detection by culture, PCR, and an enzyme immunoassay, PCR proved to be the most sensitive method for detection of virus.[12] PCR also allows characterization of the isolate via sequencing of viral genes.

Equine herpesvirus-1 (EHV-1) and EHV-4 are important, ubiquitous equine viral pathogens that cause important economic losses in the equine industry. Both are double-stranded DNA alpha-herpesviruses affecting the equine respiratory tract and can establish life-long latent infection after primary exposure. The traditional approach to the diagnosis of active EHV infection is virus isolation using nasal secretions or blood. Virus isolation often is unsuccessful, however, because herpesviruses are fragile, viral shedding is transient, and virus is present in low numbers. Many conventional PCR assays have been established to study the pathophysiology and improve the diagnosis of these viruses. One of the major drawbacks of conventional PCR analysis has been the inability to distinguish between the different viral states (eg, lytic, dead, latent). In a recent study, we followed a group of horses naturally infected with EHV-4 over a 30-day period and performed sequential real-time PCR analysis on blood and nasal swabs.[g] In an attempt to determine a possible threshold discriminating between shedding of lytic and dead virus in nasal secretions, we compared the DNA load of each sample with the corresponding mRNA load at the level of the glycoprotein B gene. We found that all DNA-positive samples that tested negative for mRNA expression were below 10^6 copies per million nasal cells. This DNA load has diagnostic implications to discriminate lytic from dead virus.

Streptococcus equi infection rarely is associated with detection difficulties when using conventional cultures in clinically affected horses. Culture of nasal swabs, nasal or guttural pouch washes, or exudate aspirated from an abscess remains the gold standard for the detection of *S equi.* Culture, however, may be unsuccessful during the incubation and early clinical phases of infection. Available PCR assays are designed to detect the DNA sequence of the *S equi* M protein gene. The test can be completed in a few hours and results may be available on the same day samples are taken. To address the viability question, real-time PCR can be run at the RNA level to confirm viability of *S equi.* In several studies, PCR proved to be up to 3 times more sensitive than culture.[13–15] PCR accompanying culture on a nasal swab or guttural pouch lavage may be used in a control program to select possible carrier animals, because PCR is capable of detecting *S equi* DNA in guttural pouch lavages 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. PCR should be considered to detect asymptomatic carriers, establish the *S equi* infection status of asymptomatic horses, and determine the success of elimination of *S equi* from the guttural pouch.

Rhodococcus equi is an important cause of chronic supportive bronchopneumonia with extensive abscessation in foals 3 weeks to 6 months of age. Culture of the organism from tracheal wash (TW) fluid currently is considered the gold standard for diagnosis.[16] However, it can be difficult to reliably grow *R equi* from a single TW sample, possibly because of prior antimicrobial administration or overgrowth by multiple pathogenic bacterial species.[17,18] Hillidge[19] reported that only 62% of foals with positive *R equi* cultures at postmortem examination and 64% of those with radiographic evidence of lung abscessation yielded *R equi* on culture of TW fluid. PCR has been evaluated in order to increase the diagnostic sensitivity of TW fluid. Strains of *R equi* isolated from sick foals uniformly contain an 85- to 90-kb plasmid that carries the gene responsible for expression of a 15- to 17-kDa antigen (vapA) of undetermined function.[20,21] Environmental strains of *R equi* not associated with disease do not contain this plasmid. Therefore, detection of the vapA gene of *R equi* in a TW fluid sample from

a foal with pneumonia can be considered diagnostic. Both culture and PCR, however, may detect environmental contaminants of *R equi* in TW fluid, but PCR has the ability to distinguish between virulent and avirulent strains. Foals, however, may have virulent or avirulent strains of *R equi* present as contaminants in their airways that are not responsible for clinical signs of pneumonia. This situation may be more likely on farms on which *R equi* problems are endemic. In a recent study evaluating the sensitivity of conventional culture, PCR and serology from 56 foals with pneumonia, PCR of TW fluid was found more sensitive and specific for the diagnosis of *R equi* pneumonia than the other 2 available diagnostic tests.[22] PCR should be used in conjunction with standard culture because of the probability of the presence of multiple bacterial pathogens and the inability of PCR to determine antimicrobial sensitivity of *R equi.* PCR with its higher sensitivity and specificity may be useful to rule out *R equi* pneumonia in culture-negative foals that have failed to improve with standard antimicrobial therapy and have clinical signs consistent with *R equi* pneumonia. It also may be useful in monitoring response to therapy and deciding when to discontinue therapy in foals that are confirmed to have *R equi* pneumonia. Our laboratory currently is evaluating the use of real-time PCR (vapA gene) in the feces of foals with pneumonia and respiratory distress. This surrogate sample can easily be collected in situations in which TW fluid collection is not possible because of the severity of the clinical signs. Pneumonic foals swallow infected sputum and the virulent bacteria can multiply in their intestines and be shed in large numbers in the feces. Preliminary results showed molecular detection of *R equi* in the feces of 3 of 4 foals with confirmed *R equi* pneumonia. The only negative sample originated from a suspected foal that had been treated with a compounded azithromycin product for 30 days before being referred.

Neurologic Pathogens

Although highly sensitive real-time molecular methods have been developed for detection of viral and protozoal genomes in the cerebrospinal fluid (CSF) of neurologic patients, these methods often are of limited value in the routine diagnosis of these diseases because viremia often is very short-lived or the pathogen has no affinity to the cells of the CSF. Consequently, the pathogen usually is no longer detectable at the onset of systemic or central nervous system signs.

Equine protozoal myeloencephalitis (EPM), caused by the protozoal apicomplexa parasites *Sarcocystis neurona* and *Neospora hughesi*, represents one of the greatest diagnostic challenges for equine practitioners. Detection of the parasite at postmorten examination is considered the gold standard. Therefore, clinical diagnosis is based on clinical findings, exclusion of other neurologic diseases, and the use of serological assays (eg, Western immunoblot, indirect immunofluorescent assay) on serum and CSF.[23–25] Molecular diagnostics also have been investigated but their sensitivity was found to be low.[26] Apparently, intact merozoites rarely enter CSF and free-parasite DNA is destroyed rapidly by enzymatic action.[27] Based on its low sensitivity we believe that PCR testing of CSF should not be recommended for routine diagnosis of EPM. In contrast, PCR testing of neural tissue has been shown to be useful as a postmortem test.[i]

Diagnosis of West Nile virus (WNV) in horses currently is based on observation of compatible clinical signs (eg, ataxia, paresis, paralysis, hyperesthesia, muscle fasciculation, seizures, fever) and 1 or more of the following: isolation of WNV from blood, CSF, or tissue, a 4-fold increase in plaque reduction neutralization test antibody titers on paired serum samples taken 2 weeks apart or the detection of IgM antibody to WNV by IgM-capture enzyme-linked immunosorbent assay (ELISA).[28,29] Given the nonspecificity of the IgM ELISA (ie, does not differentiate between disease and exposure) and the time required to serologically confirm WNV infection, alternative tests able to rapidly detect WNV in clinical specimens are important. PCR has been evaluated to investigate ante-mortem cases of suspected WNV encephalitis in horses and humans using blood. The diagnostic sensitivity of WNV PCR using either serum or whole blood was very low.[30–32] However, 57–70% of CSF samples from human beings with serologically confirmed WNV infection tested positive by real-time PCR.[30,31] The reduced ability to detect WNV in CSF or serum from patients with serologically confirmed WNV infection likely is due to the short-lived viremia in dead-end hosts and emphasizes the fact that, in order to detect WNV in CSF, the sample should be collected early during the disease process. Investigation of the sensitivity of real-time PCR on CSF from horses with WNV has not yet been reported. TaqMan PCR also has been shown to accurately identify WNV in field-collected mosquito pools, avian tissues, and human and equine brain tissue samples with a degree of sensitivity approaching that of virus isolation in Vero cells.[31–33]

Myeloencephalopathy is an uncommon presentation of EHV-1 infection and should always be considered as a differential diagnosis when a horse develops sudden neurologic signs (eg, ataxia, paresis, urinary incontinence), if multiple horses on the premises are involved or when a recent history of fever, abortion, or viral respiratory disease in the affected horse or herdmates is reported.[34] Diagnosis often is based on history, clinical signs, and xanthochromia in CSF due to vasculopathy. Attempts to isolate virus from the blood or CSF of patients often are unsuccessful because the peak of virus shedding usually has passed by the time neurologic signs appear.[35] However, affected horses can shed the virus in nasal secretions and thus represent a risk of infection for unaffected horses. This outcome recently has been reported in a hospital setting in which horses developed neurologic disease after having been exposed to horses with EHV-1 myeloencephalopathy.[j] It therefore is imperative to determine the risk of shedding in a suspected horse in order to initiate an appropriate infectious disease-control protocol. Real-time PCR, as previously shown for viral respiratory diseases, is a fast and sensitive molecular diagnostic tool and should be performed on nasopharyngeal swabs. The dilemma as to whether the virus is in a lytic or dead viral state can be addressed by using absolute quantitation or transcriptional activity of the target gene similar to the approach used for EHV-4.[g]

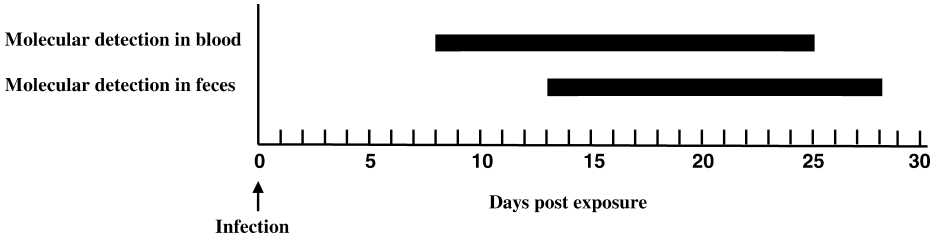

Fig 3. Molecular detection time of *Neorickettsia risticii* in blood and feces of experimentally infected horses.

Gastrointestinal Pathogens

The detection of equine gastrointestinal pathogens using conventional or molecular tests often is very 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 due to the presence of inhibitory substances in the feces that can interfere with NA extraction or amplification.[36] However, development and use of specific extraction kits[b] has improved the yield of NA from feces.[37]

Neorickettsia risticii (formerly *Ehrlichia risticii*) is the rickettsial agent responsible for Potomac horse fever (PHF), a serious enterocolitis of horses. Due to the nonspecific nature of the clinical signs, a provisional diagnosis of PHF often is based on the presence of typical clinical signs and the seasonal and geographical occurrence of the disease. A definitive diagnosis of PHF, however, should be based on isolation or detection of *N risticii* from blood or feces of infected horses.[38] Isolation of the agent in cell culture, although possible, is time consuming and not routinely available in many diagnostic laboratories. The recent development of a *N risticii*-specific real-time PCR assay has greatly facilitated diagnosis of PHF.[39] This molecular assay has contributed to 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.[40–43] NA of *N risticii* can be detected in the blood and feces of naturally or experimentally infected horses, but the detection time does not necessarily coincide between the 2 sample types (Fig 3). Based on these results, we recommend analyzing both blood and feces from suspected horses in order to enhance the chance of molecular detection of *N risticii.* Another application of the real-time PCR has been the recent description of the first equine cases of PHF from Nova Scotia using formalin-fixed and paraffin-embedded colon tissue.[44]

An emerging equine gastrointestinal pathogen, *Lawsonia intracellularis,* has been described in young horses.[45] This obligate intracellular bacterium is the causative agent of proliferative enteropathy (PE), a transmissible enteropathy known to affect a wide range of domestic and wild animal species.[46] This disease has a worldwide distribution and likely is underrecognized in horses. Antemortem diagnosis can be challenging and is based on interpreting clinical signs (eg, lethargy, weight loss, subcutaneous edema, diarrhea, colic), clinicopathologic results (eg, hypoproteinemia), ultrasonographic findings (eg, thickened small intestine), and excluding other causes of similar gastrointestinal findings.[47] Currently, culture of the organism is difficult and is not routinely offered by laboratories. Antemortem diagnosis relies on serology and PCR,[48] but these tests have not been systematically evaluated in horses. The combination of both tests will increase the chance of diagnosing the disease. Novel PCR assays, such as the real-time PCR, have increased the sensitivity of molecular detection, compared with initial conventional assays.[49] PCR has the advantage of being fast and can yield positive results in the early stage of disease, when antibodies are not yet measurable. Furthermore, the molecular assay can be used to monitor treatment and study the epidemiology of this pathogen.

Miscellaneous Pathogens

Equine granulocytic ehrlichiosis (EGE) is caused by *Anaplasma phagocytophila* (formerly *Ehrlichia equi*), a rickettsial pathogen transmitted by *Ixodes* ticks. Diagnosis often is based on awareness of the geographic area for infection, typical clinical signs, abnormal laboratory findings, and finding characteristic pathogen inclusions in the cytoplasm of neutrophils and eosinophils in a peripheral blood smear stained with Giemsa or Wright stain. Real-time PCR has been used for many years to study several aspects of the epidemiology and pathophysiology of EGE.[38,50] For clinical purposes, the material of choice is whole blood. Real-time PCR has been shown to be a very sensitive and specific tool, helping with diagnosis especially during early and late stages, when the number of organisms is too small for diagnosis by microscopy (Fig 4).[51,52]

Corynebacterium pseudotuberculosis is a common cause of external and internal abscesses in horses from arid regions of the western United States.[53] The epidemiology recently has been investigated with the help of real-time PCR and flies have been identified as potential vectors.[54,55] *C pseudotuberculosis* is easy to grow on culture and use of real-time PCR on clinical samples is restricted to specific situations (eg, culture-negative aspirates and samples of body fluids).

Additional real-time PCR assays for *Borrelia burgdorferi,* equine arteritis virus, *Leptospira* spp., *Mycobacterium* spp., and *Clostridium difficile* have been developed and are being used in the research setting.[56–60] These assays likely will be offered for diagnostic purposes when additional clinical samples from horses have been analyzed.

Conclusions and Future Diagnostic Expectations of Real-Time PCR

The number of commercially available assays continues to expand and many molecular assays continue to be de-

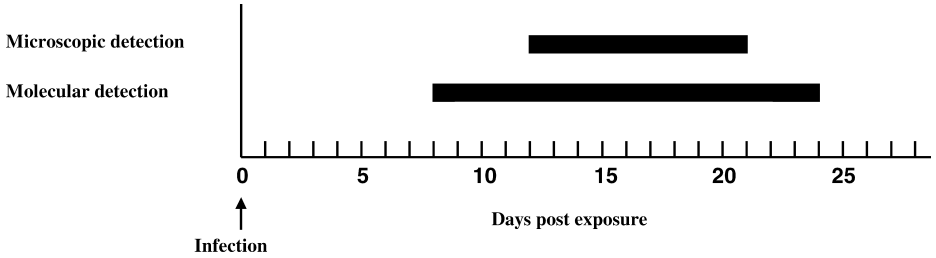

Fig 4. Microscopic and molecular detection time of *Anaplasma phagocytophila* in the blood of horses experimentally infected with *Ixodes scapularis.*

veloped in the research setting. In the meanwhile, efforts should continue to increase understanding of the strengths and limitations of these new assays. Molecular diagnostic tests may enhance diagnostic capabilities, but they should be interpreted within the clinical context and on the basis of individual laboratory performance. Extensive clinical research and strict adherence to guidelines for method validation are necessary to compare new molecular diagnostic techniques with existing methodologies, to validate new technology when comparable conventional techniques are unavailable, and to determine a method's clinical utility.

No universal standards exist for molecular assays. In 1995, the National Committee for Clinical Laboratory Standards published guidelines for molecular diagnostic methods and emphasized that the guidelines did not contain standards because molecular methods are evolving quickly.[k] The quality and partial standardization of molecular testing should be improved, including laboratory requirements for certification and proficiency surveys evaluated by an objective third party. In veterinary medicine, the American Association of Veterinary Laboratory Diagnosticians seeks to establish uniform diagnostic techniques and accepted guidelines for the improvement of diagnostic laboratory standards.[l] The probe-based real-time PCR platform is unique in providing an inherently defined standardized process that could be integrated into guidelines for molecular diagnostic testing. Standardization is necessary in order to allow comparison among laboratories. The standard should include a definition of the sample type to be analyzed, sample preparation, and PCR amplification, and reporting of quantitative results. For molecular microbiology testing, validation data should be available to detail both the analytical and diagnostic specificity and sensitivity for every assay offered. This information often is only available for assays that have been published in peer-reviewed journals.

Specimen preparation techniques are an important facet of the overall usefulness of molecular technologies used in clinical microbiology laboratories. Optimal specimen preparation will allow efficient release of NA from the clinical sample material and pathogen, while preserving the integrity of the NA, removing inhibitors, and eliminating infectivity. The use of well-established extraction kits or automated NA preparation systems is expected to provide rapid, cost-effective, and consistent results. Quality control, as for any biological test, has become a necessity for PCR assays in order to determine sample integrity, NA extraction, and PCR amplification performance. Two strategies have been proposed, spiking the patient's specimen with known target DNA or RNA or using internal controls.[61] Niesters[61] recently proposed the use of a complete nonhuman seal herpesvirus to spike human samples. In this approach, a low and fixed amount of the control virus is added to each clinical sample. This virus is coextracted with the target of clinical interest and subsequently amplified in a quantitative manner. This method monitors the combined effect of extraction (loss of sample) and amplification (inhibition) and allows more confident interpretation of the quantitative results generated for the target of clinical interest. Negative results from clinical samples, in which no target is detected, therefore can be verified with high confidence. The second approach involves the use of internal amplification controls in a single sample. These controls are amplified simultaneously with the target NA and are used to detect the presence of inhibitors and can be used to evaluate the quality and quantity of NA.[5] To assess the adequacy of specimen collection, extraction, and amplification, our laboratory uses well-conserved equine housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to assess the presence of equine cellular DNA or RNA. As an example, if a nasopharyngeal swab contains equine DNA, this finding suggests that an adequate specimen was collected and processed. In the future, standardization of reported quantitative results also will need to be addressed in veterinary medicine. So far, international standards for quantitative testing are only available for selected human pathogens.[62,63]

Probe-based real-time PCR is an established research tool to quantify infectious agents during disease and after vaccination or therapy. Adaptations of these research applications will continue to impact testing in clinical laboratories.

Footnotes

[a] BD Vacutainer Evacuated Blood Collection System, Becton, Dickinson and Company, Franklin Lakes, NJ

[b] QIAamp Kit (DNA Blood Mini Kit, RNA Blood Mini Kit, DNA Stool Mini Kit), QIAGEN, Valencia, CA

[c] ABI Prism 6700 Automated Nucleic Acid Workstation, Applied Biosystems, Foster City, CA

[d] TaqMan ABI Prism 7700 and 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA

[e] PrimerExpress, Applied Biosystems, Foster City, CA

[f] AmpErase UNG System, Applied Biosystems, Foster City, CA

[g] Pusterla N, Leutenegger CM, Berger J, et al. Equine herpesvirus 4 infection in foals: Quantitation of viral DNA in nasal secretions and peripheral blood leukocytes during an outbreak using the real-time

TaqMan PCR. Proceedings of the 21st ACVIM Forum, Charlotte, NC, 2003:387 (abstract)

[h] Directigen Flu A, Beckton, Dickinson and Company, Paramus, NJ

[i] Pusterla N, Leutenegger CM, Conrad PA, et al. Gene transcription for selected cytokines in neural tissue of horses diagnosed with equine protozoal myeloencephalitis or equine herpesvirus-1 myeloencephalopathy. Submitted for publication.

[j] Reed SM, Sofaly C, Kohn CW. Equine herpesvirus 1 myeloencephalopathy. Proceedings of the 9th IVECCS Forum, New Orleans, LA, 2003:657–659 (abstract)

[k] NCCLS: Molecular diagnostic methods for infectious disease; approved guidelines. NCCLS, Wayne, PA, 1995

[l] The American Association of Veterinary Laboratory Diagnosticians (AAVLD).

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