Use of molecular diagnostics for infectious equine pathogens
Nicola Pusterla
Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California-Davis, Davis, CA

Objectives of the presentation
- Review the basic understanding of polymerase chain reaction (PCR)
- Understanding the advantages and pitfalls of PCR
- Discuss PCR applications for respiratory, neurologic and enteric pathogens

Take home message
- Polymerase chain reaction is a nucleic acid-based amplification technique that has been described as genetic photocopying. Polymerase chain reaction allows the detection of infectious pathogens in host tissues even when only a small amount of pathogen is present.
- Polymerase chain reaction has distinct advantages as a diagnostic tool and is best used in clinical situations requiring rapid, sensitive and specific answers, which is often the case when dealing with highly infectious organisms.
- Whenever using molecular detection assays, one must keep in mind the biology of the suspected pathogen to be detected, the disease stage of the animal, the adequate specimen to be used and test to be requested. The use of a panel strategy will truly facilitate the choice of assay requested by widening the spectrum of common pathogens involved with a specific syndrome.
- Polymerase chain reaction should always be interpreted within the clinical context. If the results don’t match your clinical impression, either repeat the test or investigate the laboratory’s performance.

Introduction
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 is the best known and most successfully implemented diagnostic molecular technology to date. It can detect slow-growing, difficult-to-cultivate or uncultivable 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 quantitative PCR (qPCR), have broadened its diagnostic capabilities in clinical infectious diseases to complement and even surpass traditional methods in some situations. Automation of all components of PCR is now possible, which will decrease the risk of generating false positive results due to contamination. The diagnostic PCR applications most relevant for equine practice are presented below along with their advantages and potential pitfalls.

Respiratory pathogens
Respiratory pathogens are often contagious, and these infections must be diagnosed rapidly in order to prevent a disease outbreak and to institute the appropriate management plan. The short turn-around time and reliability of PCR makes this technology an ideal tool for the diagnosis of respiratory pathogens.

Equine influenza is commonly diagnosed by virus isolation or detection from nasopharyngeal swabs collected from horses during the early febrile stage of the disease. Although isolation of the virus is essential to allow antigenic and genetic characterization of the strain, this technology is time-
consuming and successful isolation is, to be expected at best in about 50% of the cases. In recent years new methods for virus detection, such as antigen detection enzyme-linked immunosorbent assays (ELISA) and PCR, have been described allowing a rapid diagnosis in the acute phase of infection. Polymerase chain reaction based assays have been described for the identification of equine influenza virus directly from clinical samples with higher sensitivity than virus isolation and antigen-capture ELISA. Amplification of the single stranded RNA of equine influenza viruses is performed by reverse transcription-PCR (RT-PCR) technology, using either a one-step, nested or real-time approach. The hemagglutinin, nucleoprotein and matrix genes are the common target for these PCR assays. Unfortunately, comparison of the different PCR assays is precluded by the use of different technologies, the lack of standardization among the assays, and variation in targeted genes. Nucleotide and deduced amino-acid sequences of portions of the hemagglutinin gene are now routinely used for phylogenetic characterization of outbreak strains. Further, novel qPCR assays can be used as a viable replacement for the more traditional methods of quantifying equine influenza virus in vaccine efficacy studies. Another advantage of PCR is the ability to detect non-viable virus, a situation which may occur when nasopharyngeal samples are frozen or not adequately stored and/or shipped to a diagnostic laboratory.

Equine herpesvirus (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 α-herpesviruses that affect the equine respiratory tract and can establish life-long latent infection after primary exposure. Traditionally, virus isolation has been the gold standard for diagnosing EHV-1 and EHV-4 infections using blood and nasal secretions. Virus isolation is often hampered by the fragility of the virus, intermittent viral shedding and the interference with local antibodies. Polymerase chain reaction offers an alternative to virus isolation and has proven to be a sensitive method of detecting EHV-1/-4 in respiratory secretions, peripheral blood lymphocytes and other tissues. Many conventional PCR assays have been established to study the pathophysiology and improve the diagnosis of these viruses. Conventional one-step or nested PCR assays do have inherent risks of carry-over contamination due to post-amplification steps required to detect the PCR products. Novel molecular platforms such as the qPCR have strongly reduced the risk of contamination. Polymerase chain reaction assays used in routine diagnostics are based on the detection of viral genomic DNA and are therefore unable to distinguish between lytic, non-replicating or latent virus. Alternative molecular approaches have recently been established using the qPCR to allow discrimination between the different viral states in horses naturally infected with EHV.

Streptococcus equi subsp. equi (S. equi) infection rarely is associated with detection difficulties when using conventional culture in clinically affected horses. Culture of nasal swabs, nasal or guttural pouch washes or exudates 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 phase of infection. Further, the presence of other β-hemolytic streptococci, especially S. equi subsp. zooepidemicus, may complicate interpretation of culture results. Available PCR assays are designed to detect the DNA sequence of the S. equi M protein (SeM) gene, the gene for the antiphagocytic M protein of S. equi. This gene offers enough nucleotide variations between the two S. equi subspecies to allow full discrimination in clinical specimens. The test can be completed in a few hours and results may be available on the same day samples are taken. One of the pitfalls of PCR has been its inability to distinguish between dead and live organisms, therefore, positive results have in the past been considered presumptive until confirmed by culture. Nowadays, the viability issue 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 up to three times more sensitive than culture. Polymerase chain reaction accompanying culture on a nasal swab or guttural pouch lavage may be used in a control program to select possible carrier animals. PCR should be considered to detect asymptomatic carriers, establish the S. equi infection status of asymptomatic horses and determine the success of S. equi elimination from the guttural pouch. A particular problem in the management of strep outbreaks is the lack of a suitable PCR assay to differentiate between wild-type and vaccine S. equi strains.

Rhodococcus equi is an important cause of chronic suppurative bronchopneumonia with extensive abscessation in foals three weeks to six months of age. Culture of the organism from tracheal
wash (TW) fluid currently is considered the gold standard for diagnosis. 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. Polymerase chain reaction has been evaluated in order to increase the diagnostic sensitivity of TW fluid samples. 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. 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. Polymerase chain reaction should be used in conjunction with standard culture because of the possibility that multiple bacterial pathogens are present in the lower airways and the inability of PCR to determine antimicrobial sensitivity of R. equi. Polymerase chain reaction 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. In clinical situations where the severity of the respiratory signs of the patient prevents the collection of TW fluid, feces have been shown to be a sensitive surrogate specimen for the molecular detection of R. equi.

Equine rhinitis A and B virus and equine arteritis virus, although less commonly associated with infectious upper respiratory tract diseases (IURD), are routinely detected via PCR. The role of EHV-2 and EHV-5 in nasal secretions of horses with IURD is still unclear. Due to their high prevalence in horse population and in order to avoid dilemmas with the interpretation of PCR results, the testing of γ-herpesviruses is at this time not recommended.

Neurologic pathogens

Although highly sensitive and specific PCR assays have been developed for the detection of viral and protozoal pathogens in the cerebrospinal fluid (CSF) of neurologic patients, these methods often are of limited value in the routine diagnosis of these diseases because viremia is often very short-lived or the pathogen has no affinity to the cells of the CSF. Consequently, pathogens are usually no longer detectable at the onset of systemic or CNS 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. Molecular diagnostics have also been investigated but their sensitivity was found to be low. Apparently, intact merozoites rarely enter CSF and free parasite DNA is destroyed rapidly by enzymatic action. Based on its low sensitivity, 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.

Diagnosis of West Nile virus (WNV) encephalitis in horses currently is based on observation of compatible clinical signs and the detection of serum IgM antibody to WNV by IgM-capture ELISA. Given the non-specificity of the IgM ELISA (i.e. 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. RT-PCR using either a one-step, nested or real-time approach has been evaluated to investigate ante-mortem cases of suspected WNV encephalitis in horses and humans using blood. The diagnostic sensitivity of WNV RT-PCR using either serum or whole blood was very low. However, 57 to 70% of CSF samples from human beings with serologically confirmed WNV infection tested positive by qRT-PCR. The reduced ability to detect WNV in CSF or serum from equine patients with serologically confirmed WNV infection is likely due to the short-lived viremia in dead-end hosts, and emphasizes the fact that in order to detect WNV in blood or CSF, the sample should be collected early during the disease process. Investigation of the sensitivity of RT-PCR on CSF from horses with WNV encephalitis has not yet been reported.

Equine herpesvirus myeloencephalopathy (EHM) is supported by historical and clinical findings, the presence of xanthochromia and elevated total protein concentration in CSF and the laboratory detection of EHV-1 in blood and/or nasal secretions by PCR. Because affected horses can shed the virus in nasal secretions and thus represent a risk of infection for unaffected in contact horses, it is imperative...
to determine the risk of shedding in a suspected horse in order to initiate an appropriate infectious disease control protocol. The dilemma as to whether the virus is in a lytic, non-replicating or latent state can be addressed by using 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 versus non-neuropathogenic). A single nucleotide polymorphism at position 2254 of the DNA polymerase gene (ORF 30) has been associated with a higher risk of EHM development. Rapid PCR assays have been established to allow differentiation between neuropathogenic and non-neuropathogenic strains. However, such assays have moderate specificity, since 74 to 87% of EHV-1 stains associated with EHM have been shown to be 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 using conventional tests can be challenging because these pathogens are sometimes difficult to grow. The use of fecal material for molecular diagnostics of selected pathogens has been associated with false negative results due to the presence of inhibitory substances in the feces that can interfere with nucleic acid extraction or amplification.

*Salmonella enterica* can cause enterocolitis in susceptible horses; however, infection can also be present without clinical disease in 1 to 5% of healthy horses and such horses are transient subclinical shedders. Several factors, including transportation, surgery, antimicrobial treatment, changes in diet, elevated ambient temperatures and pre-existing gastrointestinal disease, have been associated with the development of clinical salmonellosis in susceptible horses. Because these factors are often common among hospitalized horses and because the contamination of the environment by subclinical shedders poses a risk to the health of hospitalized patients and personnel, the rapid identification of horses infected with *Salmonella enterica* is of considerable importance and allows implementation of appropriate infectious disease control measures.

Microbiologic culture of feces is considered the gold standard in the detection of horses shedding *Salmonella enterica*. Time to microbiological culture and positive identification of *Salmonella* from feces by clinical laboratories requires at least 48 hours. When small numbers of *Salmonella* are present in feces, enrichment steps using selective broth are required, which prolongs the detection time even further. In recent years, PCR assays have been evaluated for the detection of *Salmonella enterica* in feces from horses admitted to veterinary hospitals. Collectively, these studies have shown that significantly more fecal samples tested positive by PCR than by microbiological culture. Today’s modern approach to screen environmental samples and feces is PCR testing of samples following a selective enrichment step (20 hr) coupled with conventional microbiological identification. Polymerase chain reaction has the advantage of having a very short turn-around-time and results can be available within 4-6 hours of having completed the selective enrichment step, which is still 2.5 days shorter than identification through conventional culture. However, conventional culture will remain the only diagnostic tool allowing serotyping and minimum inhibitory concentration (MIC) testing if needed.

*Neorickettsia risticii* is the rickettsial agent responsible for equine neorickettsiosis (EN) or Potomac horse fever, a serious enterocolitis of horses. A provisional diagnosis of EN is often based on the presence of typical clinical signs and the seasonal and geographical occurrence of the disease. A definitive diagnosis of EN, however, should be based on isolation or 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 the diagnosis of EN. Nucleic acid of *N. risticii* can be detected in the blood and feces of naturally or experimentally infected horses, but the detection period does not necessarily coincide between the two sample types. Based on these results, it is recommended to analyze both blood and feces from suspected horses in order to enhance the chance of molecular detection of *N. risticii*. 
An emerging equine gastrointestinal pathogen, *Lawsonia intracellularis*, has been described in young horses. 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. This disease has a worldwide distribution and likely is under-recognized in horses. Ante-mortem diagnosis can be challenging and is based on interpreting clinical signs, clinicopathologic results, ultrasonographic findings and excluding other causes of similar gastrointestinal findings. Currently, culture of the organism is difficult and is not routinely offered by laboratories. Diagnosis relies on serology and PCR. The combination of both tests as well as repeated fecal sampling for PCR from target animals will increase the chance of diagnosing the disease. Novel PCR assays, such as qPCR, have increased the sensitivity of molecular detection, compared to initial conventional assays. 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 success and study the epidemiology of this pathogen.

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 mostly by ECoV in a single infection without any other coinfecting agents, whereas ECoV was found exclusively in association with other coinfecting agents in sick foals. This is in agreement with coronaviruses in other species, where the virus may not have enough pathogenic potential to cause disease, but causes local immune suppression allowing secondary infections to take place more efficiently. In adult horses ECoV causes a self-limiting disease characterized by depression, anorexia, fever and less frequently changes in fecal character and colic. More epidemiological studies are needed to better understand the impact of this emerging disease.

**Conclusions**

The number of commercially available PCR assays continues to expand and many molecular assays continue to be developed 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 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. Probe-based qPCR 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.