Antemortem detection of latent infection with neuropathogenic strains of equine herpesvirus-1 in horses

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Objective—To evaluate a technique for identifying horses latently infected with neuropathogenic strains of equine herpesvirus-1 (EHV-1).

Animals—36 adult mares, 24 of which were experimentally infected as weanlings with neuropathogenic or nonneuropathogenic EHV-1.

Procedures—Mandibular lymph node (MLN) tissue was obtained from each horse via biopsy during general anesthesia. Purified DNA from MLNs was tested for EHV-1 DNA by use of a magnetic bead, sequence-capture, nested PCR assay. For MLNs that contained EHV-1 DNA, the 256-bp DNA fragments amplified via sequence-capture nested PCR were sequenced to determine the nucleotide at the polymorphic site that determines pathotype (ie, neuropathotype [G2254] or non-neuropathotype [A2254]).

Results—Latent viral DNA was detected in 26 of the 36 (72%) mares tested. Neuropathogenic and nonneuropathogenic EHV-1 genotypes were detected in the latently infected horses. In each mare previously infected with known EHV-1 pathotypes, the open reading frame 30 genotype of latent EHV-1 was identical to that of the strain that had been inoculated 4 to 5 years earlier. Latent viral DNA was detected in 10 of the 12 mares that were inoculated as weanlings with neuropathogenic strains of EHV-1. The detection rate of the sequence-capture PCR method for EHV-1 latency was double that of conventional nested or real-time PCR assays performed on the same MLN DNA preparations.

Conclusions and Clinical Relevance—The magnetic bead, sequence-capture, nested PCR technique enabled low-threshold detection of DNA from latent neuropathogenic strains of EHV-1 in MLN specimens from live horses. The technique may be used to screen horses for latent neuropathogenic EHV-1.

Outbreaks of neurologic disease caused by mutant hypervirulent strains (neuropathotypes) of EHV-1 have been reported with increasing frequency during the past several years. Equine herpesvirus-1 myeloencephalopathy has an impact on equine welfare and the potential for causing catastrophic losses in the economy of equine-related businesses because of characteristically high morbidity and case fatality rates; refractoriness to prevention by vaccination; and the ability to affect horses of all breeds, ages, and vaccination statuses.

Outbreaks of neurologic disease are thought to be initiated by viral reactivation and concomitant nasal shedding of the mutant herpesvirus by latently infected carrier horses. Development of a minimally invasive antemortem test for detection of carriers of neuropathotype strains of EHV-1 would enable a test-and-segregate approach to minimizing the risk of outbreaks resulting from reactivation of latent virus in carrier horses. In this report, the technique and performance of a test involving lymph node biopsy for antemortem detection of neuropathogenic EHV-1 infection are described.

Materials and Methods

Experimental animals—The procedure for antemortem detection of EHV-1 latency and subsequent identification of latent virus pathotype was performed in 2 categories of adult horses. The first group was composed of 24 young mixed-breed mares that had been experimentally infected as weanlings 4 to 5 years previously with neuropathogenic (n = 12 horses) or non-neuropathogenic (12) strains of EHV-1. The second group of horses was composed of 12 aged Thoroughbred mares donated by commercial horse farms to the University of Kentucky because of chronic problems with infertility, laminitis, or other forms of lameness. The 24 experimentally infected horses were kept together as an isolated group in a 10-acre grass field. None of the horses had clinical signs or laboratory evidence of EHV-1 infection at the time of MLN biopsy.

MLN biopsy procedure—Mares were sedated by IV administration of 10 mg of butorphanol tartrate solution and 500 mg of xylazine hydrochloride solution. After 5 minutes, horses were anesthetized via IV administration of 1.0 g of ketamine hydrochloride solution. With a sterile scalpel blade, a 1.5-inch incision was made through the skin overlaying palpable MLNs. An aggregate of lymph nodes weighing approximately 1.0 g was aseptically freed from surrounding connective tissue by means of blunt dissection and placed in sterile saline (0.9% NaCl) solution for transport on ice to the laboratory.

Preparation of DNA from MLN tissue—For isolation of cellular DNA from MLN tissues, 750 mg of tissue was finely
minced with a sterile disposable scalpel blade, homogenized in 5.0 mL of lysis buffer solution (10 mM Tris-HCl [pH 8.0]; 100 mM EDTA; 0.5% SDS), and incubated for approximately 24 hours at 30°C with protease K (concentration, 100 µg/mL) and ribonuclease A (concentration, 20 µg/mL). Purification of DNA from the digested cell lysate proceeded via 2 extractions with phenol:chloroform:isoamyl alcohol (25:24:1) solution and precipitation of DNA from the aqueous phase with ammonium acetate and isopropanol. The DNA precipitate was collected by use of centrifugation, rinsed twice with 70% ethanol, dissolved in 2.0 mL of sterile water, and quantitated by measuring spectrometric absorbance at 260 nm.

Detection of EHV-1 DNA by PCR assay—The DNA purified from MLN tissue was tested for EHV-1 DNA by use of the magnetic bead, sequence-capture, nested PCR method. The procedure involves oligonucleotide-hybridization enrichment and biotin-streptavidin magnetic bead technology for capture of EHV-1 DNA and was developed for detection of rare, low-abundance sequences below the detection threshold of conventional nested PCR assays. Two thousand four hundred micrograms of each MLN DNA preparation was analyzed for EHV-1 DNA. Mandibular lymph node DNA was digested overnight at 37°C with 7,500 units of Bgl-II restriction endonuclease, denatured by boiling for 10 minutes, and hybridized for 24 hours at 60°C with 24 pmol of a biotinylated capture oligonucleotide (Appendix) complementary to the EHV-1 gene sequence encoding the viral DNA polymerase (ie, ORF30). Six hundred micrograms of streptavidin-coated paramagnetic beads was added to the hybridization mixture, and the mixture was incubated for 24 hours at 4°C. After washing, beads were dispersed into six 100-µL PCR reaction mixtures containing 200 µM each of deoxyribonucleoside triphosphates, 2.5 mM MgCl₂, 5 units of Taq DNA polymerase, and 0.25 µM ORF30 primers designed to amplify a 256-bp gene fragment that encompasses the site of the mutation associated with neuropathotype strains of EHV-1 (ORF30 G2254). The biotinylated capture oligonucleotide and the amplification primers used are specific for EHV-1 and do not cross-amplify DNA from EHV-4. Amplification via PCR was performed according to the following specifications: initial denaturation at 94°C for 3 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 65°C for 1 minute; and a final extension at 65°C for 10 minutes. The second round of PCR consisted of amplification of 2 µL of a 1:10 dilution of the first PCR product with a nested set of primers and the same thermocycler specifications. After the second round of PCR amplification, reaction mixtures were analyzed for a 256-bp DNA fragment by use of electrophoresis through 1.5% agarose gel.

Peripheral blood mononuclear cells collected at the time of MLN biopsy from each of the 36 horses were also assayed for EHV-1 DNA with an identical amount (2.4 mg) of PBMC DNA and the same sequence-capture, nested PCR procedure.

To compare detection sensitivity of the magnetic bead, sequence-capture PCR method with that of conventional nested and real-time PCR assays, DNA preparations from the MLN of each of the 36 horses were also tested via the other 2 formats. The DNA purified from PBMC was tested for EHV-1 DNA with the same sequence-capture, nested PCR procedure.

Reverse transcription-PCR assay—To rule out the possibility of active (rather than latent) EHV-1 infection in the horses with positive PCR assay results, RNA was isolated from MLN tissue of 6 of the mares with positive results by means of extraction with total RNA isolation reagent and subjected to magnetic bead-based, sequence-capture reverse transcription–nested PCR use of the same capture probe and PCR primers as described.

Pathotype identification of latent EHV-1—For MLNs with positive results for EHV-1 DNA, the 256-bp fragments amplified by sequence-capture, nested PCR were sequenced to identify the base at the polymorphic site (ie, position 2254 of ORF30) that allows determination of the pathotype of the latent herpesvirus strain (ie, neuropathotype [G2254] or non-neuropathotype [A2254]). The 256-bp amplicons were purified by use of a DNA purification resin. Nucleotide sequence determination of the amplicons was performed at the University of Kentucky core sequencing facility by use of an automated fluorescence sequencer and cycle sequencing technology.

Results

Biopsy specimens of MLNs collected from each of the 36 mares were tested for EHV-1 DNA. Results of tests for detection of EHV-1 latency and identification of the pathotype of each latent virus strain were summarized (Table 1). Mandibular lymph node tissue from 26 of the 36 (72%) study mares contained latent EHV-1 DNA. Equine herpesvirus-1 DNA was detected in 18 horses with positive results for EHV-1 by the conventional nested and real-time PCR assays. One of these 8 horses had the neuropathotype strain, and 7 had non-neuropathotype strains of EHV-1.

<table>
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<th>Table 1—Frequency (%) of detection of latent EHV-1 DNA in MLNs by group in 36 horses.</th>
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<td><strong>Mare group</strong></td>
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<tr>
<td>Experimentally infected with neuropathogenic EHV-1</td>
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<td>Experimentally infected with non-neuropathogenic EHV-1</td>
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<td>Donated with unknown EHV-1 infection history</td>
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*The same horses had positive results for EHV-1 by the conventional nested and real-time PCR assays. †One of these 8 horses had the neuropathotype strain, and 7 had non-neuropathotype strains of EHV-1.
to-G2254 mutation associated with neuropathogenic potential of EHV-1 strains. A single-point mutation (A-to-G transition) in the 256-bp ORF30 gene fragment that encompasses the site of the A2254-to-G2254 mutation unique to the ORF30 gene in neuropathogenic strains of EHV-1 creates a novel restriction fragment length polymorphism in the 256-bp DNA fragment of ORF30 amplified by PCR. Agarose gel electrophoretic analysis of DNA fragments generated by Sal I digestion of the 256-bp amplicon provided an alternative and simple approach (compared with DNA sequencing) for differentiation of neuropathogenic from non-neuropathogenic strains of EHV-1 (Figure 1). The results also revealed that the quantitative burden of latent viral DNA in MLNs varied among horses (Figure 2). In some horses, EHV-1 DNA was amplified from all 6 sample replicates, whereas in others, as few as 1 or 2 replicates contained detectable viral DNA.

Discussion

The technique described in the present study permitted determination of whether certain horses carried DNA of neuropathogenic strains of EHV-1 latently sequestered in cells of the respiratory tract lymph nodes. A single-point mutation (A-to-G transition) in EHV-1 ORF30 that encodes the catalytic subunit of the viral DNA polymerase is highly associated with the viral attribute of neuropathogenicity.20 The recent increase in outbreaks of neurologic disease caused by such EHV-1 mutants has generated a practical need for identifying latent carriers of mutant neuropathogenic strains of the virus.

Detection of rare herpesviral DNA sequences among large quantities of host cellular DNA is of key importance in investigations of EHV-1 latency. Both conventional nested PCR and real-time PCR techniques are limited by the sample mass that can be tested (microgram quantities), reducing their detection of ultra-low–frequency target molecules. The present report describes the development and validation of an alternative enhanced-sensitivity PCR methodology (sequence-capture, nested PCR) and its usefulness in antemortem detection of latent EHV-1 DNA in biopsy specimens of MLN tissue. The 3 steps of the technique are enrichment of EHV-1 DNA from a large sample mass of cellular DNA by means of hybridization to a biotinylated EHV-1 allele-specific oligonucleotide, capture of the oligo–EHV-1 hybrids on streptavidin-coated paramagnetic beads, and amplification of the captured EHV-1 DNA by use of nested PCR. A final step of nucleotide sequencing or restriction fragment length polymorphism of the amplified DNA permits pathotype identification of the latent virus. The settings for temperature, time, and concentration during the procedure were optimized for detection of latent EHV-1 DNA in MLN tissue. Results of preliminary studies
designed to assess the detection threshold of the sequence-capture PCR method by testing for low numbers of EHV-1 DNA molecules in a dilution series indicated that the threshold for detection was limited only by the volume of sample available for analysis. The technique used in the study enables detection of EHV-1 DNA molecules at very low concentrations in large amounts (ie, in milligram quantities) of cellular DNA. A procedure for antemortem screening of groups of horses for carriers of latent neuropathogenic strains of EHV-1 has not previously been available.

To ascertain whether the technique could be used for screening horses for latent EHV-1 infections and for determining the pathotype of detected latent virus, MLNs of 24 experimental horses with previous exposure to known pathotypes of EHV-1 and of 12 horses with unknown EHV-1 infection histories were tested. Results of those experiments indicate that the procedure is a minimally invasive, practical, and low-threshold technique for detecting latent neuropathotype EHV-1 in live horses. Furthermore, the studies confirm that survivors of EHV-1 neurologic disease may become latently infected carriers of the hypervirulent neuropathotype strains.

Two unexpected findings resulted from the study. First, in 50% of the horses in which latent EHV-1 infection was detected in the MLN via sequence-capture nested PCR, the latent virus load was below the threshold of detection for conventional nested and real-time PCR assays. Second, EHV-1 DNA was not detected in the PBMCs of horses whose MLNs contained latent virus. Because only 6 μg of MLN DNA was screened by the conventional and real-time PCR assays (ie, six 1-μg replicates), 400 individual reactions would be required to achieve a level of detection similar to that of the sequence-capture PCR technique. Thus, results of studies that use only conventional PCR techniques or test only peripheral blood leukocytes as means of detecting latent EHV-1 infections would underestimate the true prevalence of both herpesviral pathotypes in the study sample. Regarding the nature of the cell type in which latent EHV-1 infection is established, data suggest that a sessile, noncirculating cell immobilized within lymph nodes may harbor the latent viral DNA molecules. Another question raised by results of the present study is whether EHV-1 DNA detected in PBMCs of horses whose MLNs contained latent virus immobilized by the volume of sample available for analysis. The technique used in the study enables detection of EHV-1 DNA molecules at very low concentrations in large amounts (ie, in milligram quantities) of cellular DNA. A procedure for antemortem screening of groups of horses for carriers of latent neuropathogenic strains of EHV-1 has not previously been available.

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