Papers

Prevalence of equine herpesvirus type 1 in trigeminal ganglia and submandibular lymph nodes of equids examined postmortem

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The objective of this study was to detect and characterise the biovar of equine herpesvirus type 1 (EHV-1) from submandibular lymph nodes (SMLNs) and trigeminal ganglia from 153 equids undergoing routine postmortem examination for various medical and surgical reasons. A combination of nucleic acid precipitation and preamplification steps was used to increase the analytical sensitivity of the analysis. The presence of latent EHV-1 was determined when tissue samples were PCR-positive for the glycoprotein B (*gB*) gene and the DNA polymerase (ORF 30) gene of EHV-1 in the absence of detectable late structural protein gene (*gB* gene) mRNA. The SMLNs of five of the study animals (3.3 per cent) were PCR-positive for the *gB* gene of EHV-1. Two SMLNs carried a latent neurotropic strain of the virus, whereas three SMLNs were PCR-positive for both neurotropic and non-neurotropic EHV-1. A total of 30 trigeminal ganglia collected from 19 horses were PCR-positive for the *gB* gene of EHV-1. Nine trigeminal ganglia harboured either latent non-neurotropic or neurotropic EHV-1 strains. Twelve trigeminal ganglia contained both latent neurotropic and non-neurotropic EHV-1. The prevalence and distribution of EHV-1 biovars among the study horses appeared to be influenced by their breed and the type of tissue tested.

EQUINE herpesvirus type 1 (EHV-1) is a ubiquitous viral pathogen responsible for abortions, neonatal deaths, respiratory disease and myeloencephalopathy in equids (Van Maanen 2002). EHV-1 circulates in horse populations via subclinical or clinical infection following primary infection or reactivation (Slater 2007). The virus is shed during a primary lytic infection or following reactivation from a latent state in both the lymphoreticular and neurological tissues of horses (Slater 2007). Latency and reactivation are important features of the epidemiology of EHV-1 infections.

The reported prevalence of latent EHV-1 infections varies. A study of submandibular lymph nodes (SMLNs) from mature horses in Kentucky, USA, detected latent infection in eight of 12 horses (66 per cent) with an unknown EHV-1 infection history and 18 of 24 horses (75 per cent) that had been experimentally infected as weanlings (Allen 2006). In a large study involving 132 adult thoroughbred mares undergoing routine postmortem examination, latent EHV-1 was documented in the SMLNs of 71 (54 per cent) (Allen and others 2008). That study further determined that 58 (82 per cent) of the latently infected horses harboured a non-neurotropic biovar of EHV-1, 10 (14 per cent) harboured both biovars and only three (4 per cent) were latently infected with a neurotropic biovar. The prevalence of latent EHV-1 infection may be influenced by the geographical region, horse population, management practices, sensitivity of testing and other factors. To gain more information on the prevalence and biovar distribution of EHV-1 latency in horses from California, USA, this study used real-time PCR to detect sequences of neurotropic and non-neurotropic EHV-1 biovars from trigeminal ganglia and SMLNs.

Materials and methods

A total of 147 horses, four mules and two donkeys euthanased for various medical and surgical reasons from April 2007 to June 2008 were enrolled in the study. Care was taken not to enrol horses with signs of acute respiratory or neurological disease that could be due to EHV-1 infection. Several breeds were included, including quarter horse (47 horses), thoroughbred (36), Arabian (20), American paint horse (10), warmblood (9), pony (5), Morgan (4), Percheron (4), Tennessee walking horse (4), paso fino (2), standardbred (2), mustang (2), and fox trotter (2). There were 75 geldings, 54 mares, 11 stallions and 13 foals. The age of the horses, mules and donkeys ranged from two months to 39 years (median 13.0 years).

SMLNs and trigeminal ganglia were collected from each study animal during routine postmortem examination. Disposable sterile instruments were used to retrieve each tissue sample and place them into sterile plastic bags, which were immediately stored at −80°C until further processing. In addition, whole blood samples and nasal swabs (Sterile Rayon Tipped Applicators; Puritan Products) were collected from 68 animals before euthanasia. Nucleic acid extraction from whole blood and nasal secretions was performed on the day of collection, using an automated nucleic acid extraction system according to the manufacturer's recommendations (CAS-1820 X-tractor Gene; Corbett Life Science). All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, and owner consent was requested before the samples were collected.

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TABLE 1: PCR results for equine herpesvirus type 1 (EHV-1) glycoprotein B (*gB*) gene and ORF 30 gene in submandibular lymph nodes and trigeminal ganglia from EHV-1-positive hospitalised equids undergoing routine postmortem examination

Age (years)	Breed	Sex	Submandibular lymph node			Trigeminal ganglia		
			gB gene	ORF 30 (N_{752})	ORF 30 (D_{752})	*gB* gene	ORF 30 (N_{752})	ORF 30 (D_{752})
2	Thoroughbred	Male	+	–	+	–	R–, L–	R–, L–
3	Thoroughbred	Male	+	+	+	+	R+, L+	R+, L+
3	Thoroughbred	Female	–	–	–	+	R–, L–	R+, L+
3	Thoroughbred	Gelding	–	–	–	+	R+, L–	R–, L–
4	Thoroughbred	Gelding	+	+	+	–	R–, L–	R–, L–
7	Thoroughbred	Gelding	–	–	–	+	R+, L+	R–, L–
12	Thoroughbred	Male	–	–	–	+	R+, L+	R+, L+
14	Thoroughbred	Female	–	–	–	+	R–, L–	R+, L–
20	Thoroughbred	Female	–	–	–	+	R+, L+	R+, L+
20	Thoroughbred	Gelding	–	–	–	+	R–, L–	R–, L+
29	Thoroughbred	Gelding	–	–	–	+	R–, L–	R+, L+
11	Quarter horse	Female	+	+	+	–	R–, L–	R–, L–
26	Quarter horse	Gelding	–	–	–	+	R+, L–	R–, L–
29	Quarter horse	Gelding	+	–	+	–	R–, L–	R–, L–
20	Mule	Gelding	–	–	–	+	R+, L–	R–, L–
25	Mule	Gelding	–	–	–	+	R+, L–	R–, L–
2	Donkey	Male	–	–	–	+	R–, L–	R+, L+
35	Donkey	Female	–	–	–	+	R+, L+	R–, L–
26	Arabian	Gelding	–	–	–	+	R+, L+	R+, L+
35	Arabian	Gelding	–	–	–	+	R–, L–	R–, L+
10	Percheron	Female	–	–	–	+	R+, L–	R–, L–
25	Standardbred	Female	–	–	–	+	R+, L+	R+, L+
30	American paint	Female	–	–	–	+	R+, L+	R+, L+

+ PCR-positive result, – PCR-negative result, L Left, ORF 30 (N_{752}) non-neurotropic biovar, ORF 30 (D_{752}) neurotropic biovar, R Right

The tissue samples were thawed and a 500 mg piece of SMLN or trigeminal ganglia was collected in a sterile fashion under a biosafety cabinet and placed into a deep-well plate (Masterblock; Greiner Bio-One) containing 500 µl DX binding buffer (Applied Biosystems); two 4 mm diameter stainless steel beads were added, and the tissue samples were homogenised in a grinder (Geno Grinder 2000; SpexCertiprep) for two minutes at 1000 strokes per minute. Samples were stored at –20°C for one hour to reduce foam, and a protein digest was then completed at 56°C for 30 minutes. Nucleic acid was extracted from the tissue lysates, using an automated nucleic acid workstation according to the manufacturer's instructions (CAS-1820 X-tractor Gene; Corbett Life Science).

Each purified nucleic acid sample underwent an additional precipitation and PCR preamplification step. DNA was precipitated using 100 µl of purified sample, 6 µl 5M sodium chloride (Ambion), 10 µl 5 mg/ml glycogen (Fermentas) and 300 µl absolute ethanol (Gold Shield Chemical) in a 1.5 ml microcentrifuge tube. The mixture was mixed by inverting the tube several times and was stored at –20°C overnight. The tubes were then centrifuged at 4°C at 16,000 *g* for 15 minutes. The supernatant was removed, and the pellet was washed by resuspending it in 200 µl 70 per cent ethanol. The last step was repeated and the pellet was dried at room temperature for five to 10 minutes. Finally, the pellet was resuspended in 20 µl water. Half of the nucleic acid sample (10 µl) was stored at –20°C until after the analysis of genomic DNA (gDNA).

Total RNA was purified from the remaining half of the nucleic acid sample as follows: 10 µl of each nucleic acid sample was digested with gDNA WipeOut Buffer (Quantitect Reverse Transcription Kit; Qiagen) for two minutes at 42°C to remove viral gDNA. Before complementary DNA (cDNA) synthesis, 1 µl of each sample was tested for gDNA background using the EHV-1 glycoprotein B (gB) gene to ensure complete removal of the target (Pusterla and others 2009). Only samples negative for EHV-1 gB were processed for cDNA synthesis, and positive samples were redigested and retested. cDNA from each sample was synthesised using the Quantitect Reverse Transcription Kit with the following modifications: 10 µl nucleic acid was DNase digested with 1 µl gDNA WipeOut Buffer (Qiagen) by incubation at 42°C for two minutes and then briefly centrifuged. Then, 0.5 µl Quantitect Reverse Transcriptase (Qiagen), 2 µl Quantitect RT buffer (Qiagen), 0.5 µl RT Primer Mix (Qiagen), 0.5 µl 20 pmol random primers (Invitrogen) were added, and the reaction mixture was brought up to a final volume of 20 µl, incubated at 42°C for 40 minutes, inactivated at 95°C for three minutes and briefly centrifuged. Finally, 80 µl water was added and mixed thoroughly.

The resulting cDNA samples were stored at –20°C. Precautions taken to minimise contamination during the precipitation and preamplification steps included performing all pipetting steps in a laminar flow cabinet and including positive and negative EHV-1 DNA controls. In addition, swabs were taken from centrifuges, laminar flow cabinet and counter tops, and assayed for the gB gene of EHV-1 by real-time PCR in order to assess potential contamination (Pusterla and others 2009).

Preamplification was done using the Advantage 2 Polymerase Mix (Clontech Laboratories) containing the target primers for the gB gene (forward primer 5′-TATACTCGCTGAGGATGGAGACTTT-3′, reverse primer 5′-TTGGGGCAAGTTCTAGGTGGTT-3′) and the ORF 30 gene of EHV-1 (forward primer 5′-ATCTGGCCGGGCTTCAAC-3′, reverse primer 5′-GGTCACCCACCTCGAACGT-3′) as well as the target primers for the housekeeping gene and equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH) gene (forward primer 5′-AAGTGGATATTGTCGCCATCAAT-3′, reverse primer 5′-AACTTGCCATGGGTGGAATC-3′) in a 50 µl total volume as follows (Pusterla and others 2009): each reaction contained 5 µl 10X buffer, 1 µl 10mM dNTPs, 1 µl Advantage 2 Polymerase, 1 µl of each target primers at 10 pmol, 20 µl DNA and 17 µl water. The primer mix was amplified in a DNA Engine Dyad thermocycler (Peltier Thermal Cycler; Bio-Rad Laboratories) under the following conditions: one minute at 94°C, followed by 25 cycles of 15 seconds at 94°C, 15 seconds at 55°C and 40 seconds at 70°C, followed by five minutes at 70°C.

All samples (DNA from whole blood and nasal secretions and precipitated and preamplified DNA and cDNA from tissue samples) were assayed for the presence of the gB and the DNA polymerase (ORF 30) gene of EHV-1 and the housekeeping gene eGAPDH, using previously reported real-time TaqMan PCR assays (Pusterla and others 2009). Latency for EHV-1 was assumed when total nucleic acid samples were positive for the gB and ORF 30 sequences by PCR and mRNA for late structural protein gene (gB) was not detected by real-time PCR. On the basis of the single nucleotide polymorphism at nucleotide position 2254 of the ORF 30 gene of EHV-1, biovars were reported as non-neurotropic (ORF 30 [N_{752}]) or neurotropic (ORF 30 [D_{752}]) biovars (Nugent and others 2006).

Results

All of the 68 uncoagulated blood samples and nasal swabs were PCR-negative for gB and ORF 30 gene sequences. All tissue samples passed quality control following precipitation and preamplification based on established eGAPDH values (cycle threshold range nine to 13). The SMLNs of five (3.3 per cent) of the study animals were PCR-positive for both gB and ORF 30 gene sequences. Test-positive SMLNs were from three thoroughbreds and two quarter horses aged between two and 29 years (median 4.0 years). Two SMLNs carried a latent neurotropic strain of the virus, and three SMLNs tested PCR-positive for both neurotropic and non-neurotropic virus strains (Table 1). Thirty trigeminal ganglia collected from 19 horses were PCR-positive for gB and ORF 30 gene sequences of EHV-1. Eleven horses tested PCR-positive for the gB gene of EHV-1 in both trigeminal ganglia, and eight horses tested PCR-positive in only one trigeminal ganglia. These included nine thoroughbreds, two Arabians, two mules, two donkeys, one quarter horse, one Percheron, one standardbred and one American paint horse, ranging in age between two and 35 years

(median 20.0 years). Nine trigeminal ganglia harboured either latent non-neurotropic or neurotropic EHV-1 strains; 12 trigeminal ganglia were PCR-positive for both latent neurotropic and non-neurotropic EHV-1 strains (Table 1). Four of five horses with EHV-1 PCR-positive SMLNs had no detectable EHV-1 in the trigeminal ganglia. Only one horse had both PCR-positive SMLNs and trigeminal ganglia of a similar biovar. Evidence of gB mRNA was not found in any of the SMLNs and trigeminal ganglia, which ruled out lytic viral infection. All environmental swabs collected from centrifuges, laminar flow cabinet and counter tops tested PCR-negative for the gB gene of EHV-1.

Discussion

Conventional PCR and co-cultivation have been used to evaluate the prevalence of latent EHV-1 in different tissues, with the caveat that these techniques lack sensitivity to detect latent virus present in a low proportion of cells. Techniques are available that concentrate nucleic acid to a level that allows the detection of even just a single target gene from a tissue sample (Allen 2006, Pusterla and others 2008). Strict precautions are necessary when such highly sensitive techniques are used in order to prevent contamination with EHV-1 DNA from the laboratory equipment and environment, and between individual samples. One limitation of such techniques is the small size of the tissue sample processed (500 mg) compared to the whole organ, and the inability to detect EHV-1 DNA if the virus is not evenly distributed within the tissue. To determine whether sampling error might have affected the results of this study, up to 10 different pieces of tissue were analysed from each of a subset of 10 PCR-negative and 10 PCR-positive SMLNs or trigeminal ganglia. The PCR results were identical for all tissue pieces from the same organ, suggesting that latent EHV-1 is evenly distributed in organs with latent infection.

Latent EHV-1 has been detected in both lymphoid and neural tissues (Slater and others 1994). The preferred cell in which latency is maintained is the CD8+ T lymphocyte (Slater 2007). In the present study, the prevalence of latent EHV-1 in SMLNs and trigeminal ganglia was 15 per cent (23 of 153 horses), with a higher prevalence in trigeminal ganglia (12.4 per cent) than SMLNs (3.3 per cent). The reason for the discrepancy in prevalence between the two tissue types is not clear. No study has looked at the affinity of infectious EHV-1 for SMLNs and trigeminal ganglia, the ability of the host to clear infection from these sites or the relative ability of these organs to sustain latency. Although the number of latently infected lymphocytes declines over time, it appears that the population of latently infected neurons is assumed to be stable due to their longevity (Slater 2007). In the horse population studied, the location of latent EHV-1 seemed to be mutually exclusive to either the SMLNs or the trigeminal ganglia, with only one horse testing PCR-positive for EHV-1 in both tissue types. Of interest is the observation that horses with EHV-1 PCR-positive SMLNs were generally younger than horses with EHV-1 PCR-positive trigeminal ganglia, although this difference was not statistically significant (Mann-Whitney U test, $P>0.05$). EHV-1 latency in trigeminal ganglia may be more likely to occur in older horses following repeated infections or reactivations.

The results of this study are in sharp contrast to those of a study by Allen and others (2008) reporting the prevalence of EHV-1 biovars in the SMLNs of 132 thoroughbred broodmares from central Kentucky, using a magnetic bead-based, sequence-capture nested PCR technique. Latent EHV-1 DNA was detected in the SMLN tissues of 54 per cent of the mature horses submitted for postmortem examination, compared to the 3.3 per cent determined in the present study. Although the detection techniques used in the two studies were different, both have a similar analytical sensitivity of one EHV-1 target gene per 500 mg of extracted tissue. Differences in horse populations, geographical origin and management practices may have accounted for the apparent differences in prevalence. The low prevalence of latent infection in the present study is compatible with previous observations that EHV-1 viral shedding and/or viraemia were uncommon in critically ill horses hospitalised in the William R. Pritchard Veterinary Medical Teaching Hospital (VMTH) at the University of California, Davis (Carr and others 2008). Together, these studies suggest that the prevalence of latent EHV-1 is low in the population of horses admitted to the VMTH. An apparent breed and species predilection may be associated with latent EHV-1 infection in the study population, as only 6 per cent of quarter horses (three of 47) were infected with latent EHV-1 compared with 30 per cent of thoroughbreds (11 of 36) and 67 per cent of mules/donkeys (four of six). The apparent high prevalence of latently infected mules and donkeys needs to be interpreted with caution, due to the small number of these animals in the study.

In the study by Allen and others (2008) it was found that 81 per cent of SMLNs harboured only a non-neurotropic biovar; 14 per cent harboured both non-neurotropic and neutropic biovars, and 4 per cent harboured only a neurotropic biovar. In the present study, 44 per cent of EHV-1-positive tissues harboured DNA of both neurotropic and non-neurotropic biovars, 27 per cent harboured a neurotropic biovar and 27 per cent harboured a non-neurotropic biovar. The apparent difference in distribution of EHV-1 biovars in the study by Allen and others (2008) and the present study may be related to the horse populations studied or variations in tissue tropism for latent EHV-1 (that is, SMLNs v trigeminal ganglia). Future studies involving different horse populations from different geographical origins are needed to determine the overall prevalence of latent EHV-1 and gain a better understanding of factors underlying the differences in prevalence reported in different studies.

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