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Characterization of viral loads, strain and state of equine herpesvirus-1 using real-time PCR in horses following natural exposure at a racetrack in California

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Abstract

The objective of this study was to determine viral loads, strain (neuropathogenic versus non-neuropathogenic) and state (lytic, non-replicating, latent) of equine herpesvirus-1 (EHV-1) by real-time polymerase chain reaction (PCR) in the blood and nasopharyngeal secretions of adult horses following natural exposure. The index case, a 4-year-old Thoroughbred gelding with confirmed EHV-1 myeloencephalopathy, as well as potentially exposed horses, were sampled over a period of 3 weeks. The study population comprised of 39 adult Thoroughbred horses and 35 adult "pony" and outrider horses of various breeds housed at a racetrack in Northern California. Blood samples and nasopharyngeal secretions (NPS) from all horses were tested on several occasions for EHV-1 DNA viral loads, targeting the *glycoprotein B* (*gB*) gene, viral strain, targeting the *ORF 30* gene, and transcriptional activity of EHV-1, targeting the *gB* gene and latency-associated transcripts (LATs).

Viral loads and transcriptional activity of the *gB* gene declined rapidly in the index case following antiviral treatment. The prevalence of EHV-1 infection in NPS determined by PCR slowly decreased over the 22 day study period from 25% to 14%. The initial surveillance showed multiple clusters of exposure, one associated with the index case and two related to horses that had recently returned from a different racetrack. Viral strain differentiation showed that only two horses (the index case and a neighboring horse) were infected with only a neuropathogenic strain, while all other horses were infected with either a non-neuropathogenic strain or were dually infected with both neuropathogenic and non-neuropathogenic strains. In most cases, the virus was present in either a lytic or a non-replicating form, while latent virus was found in blood and NPS much less frequently. The molecular approach used in this study showed promise for assessing the risk of exposing other horses to EHV-1 and for studying viral kinetics in infected horses.

Keywords: Equine herpesvirus-1; Viral loads; Viral strain; Viral state; Real-time PCR

Introduction

Equine herpesvirus-1 (EHV-1) is an important, ubiquitous equine viral pathogen that causes significant economic losses to the equine industry and produces well-documented syndromes of respiratory disease, abortion, neonatal foal death and myeloencephalopathy (Van Maanen, 2002). As with other alpha-herpesviruses, primary exposure is often followed by a life-long, latent infection in recovered horses. Periodic reactivation of latent infections

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can lead to viral shedding and horizontal transmission (Edington et al., 1985) so early recognition of the disease is crucial for implementation of management practices that decrease the risk of exposure of susceptible horses. This has been exemplified in recent outbreaks at riding schools, racetracks and veterinary hospitals throughout North America and Europe.

Traditionally, virus isolation from a nasopharyngeal swab or blood has been the 'gold standard' for the diagnosis of viral shedding or viremia, respectively. Recently, polymerase chain reaction (PCR) assays, that are both more sensitive and rapid, have largely replaced the time-consuming procedure of virus isolation (Sharma et al., 1992; Borchers and Slater, 1993; Allen and Breathnach, 2006; Hussey et al., 2006; Pusterla et al., 2006). Research groups have recently identified a region of variation in the genome of different EHV-1 strains, which correlated directly with their ability to cause neurological disease (Nugent et al., 2006). The sequence variation occurs in the *DNA polymerase* gene (*ORF 30*) of the virus, which is involved in initial viral replication within infected cells.

PCR assays based on *ORF 30* have recently been developed and have for the first time allowed differentiation of neuropathogenic from non-neuropathogenic strains in samples submitted from field outbreaks (Allen, 2007; Leutenegger et al., in press). This wild type nucleotide A_{2254} is present in approximately 95% of non-neuropathogenic isolates, whereas approximately 86% of the neuropathogenic isolates encoded G_{2254}, leading to an amino acid change at $N_{752}D$ (Nugent et al., 2006). It seemed obvious, therefore, that additional single nucleotide polymorphism (SNP), alone or more likely in combination, may increase the accuracy of future minor groove binder (MGB) probe-based diagnostic TaqMan assays.

The increased use of PCR for the molecular detection of EHV-1 in clinical practice has presented new dilemmas with regard to how test results are interpreted and used by equine practitioners and regulatory veterinarians, since routine PCR assays are unable to differentiate between replicating (lytic), non-replicating or latent virus. Quantitative real-time PCR analysis at both the DNA and mRNA level provides the possibility of differentiating between different viral states. The key in using molecular tools to assess the different viral stages relates to the well known biology of the EHV-1 and its highly controlled and conserved cascade of gene expression (Slater, 2007). Although the molecular detection of a late gene, such as the envelope *glycoprotein B* (*gB*) gene, only reflected the presence of the genome, the combination of this information with the detection or absence of specific transcripts present during replication or latency may distinguish between viral replication and latency, respectively (Pusterla et al., 2005).

The objective of the current study was to determine viral loads, strain (neuropathogenic versus non-neuropathogenic), and state (lytic, non-replicating, latent) of EHV-1 by real-time PCR in blood and nasopharyngeal secretions (NPS) collected from adult horses following natural exposure.

Materials and methods

Index case

The index case was a 4-year-old Thoroughbred gelding from a racetrack in northern California that was presented to the Veterinary Medical Teaching Hospital (VMTH) at the University of California at Davis School of Veterinary Medicine, late in the afternoon of 28 December 2006, for acute onset of ataxia and urinary incontinence following a 3 day period of fever. The horse was received into the Equine Isolation unit, where he remained throughout the hospital stay.

On presentation, the horse was afebrile, but showed profound neurological deficits characterized by a grade 4/5 ataxia and severe proprioceptive deficits involving all four limbs, poor tail and anal tone, distended bladder on rectal examination and urinary incontinence. The diagnosis of equine herpesvirus myeloencephalopathy (EHM) was made based on the clinical signs, the results of laboratory analysis of cerebrospinal fluid (lymphocytic pleocytosis with mild mononuclear reactivity, mild erythrophagia, xanthochromia, and markedly increased protein concentration of 279 mg/dL), and the detection of EHV-1 in whole blood and nasopharyngeal secretions by real-time PCR.

Treatment was initiated 4 h post-admission, after collection of samples for molecular detection of EHV-1, and included the administration of intravenous crystalloid fluids (2 mL/kg bodyweight [BW]/h), flunixin meglumine (1.1 mg/kg BW intravenously [IV] q12 h), valacyclovir (30 mg/kg BW per os [PO] q12 h), vitamin E (5000 IU PO q24 h), and ceftiofur (2.2 mg/kg BW IV q12 h), as well as supportive (bladder catheterization) and nutritional care. Uncoagulated blood and nasopharyngeal swabs (Fox Converting Swabs) were collected every 12 h to study the viral kinetics of EHV-1 during the course of antiviral treatment.

Neurological deficits stabilized rapidly and the horse remained standing. The horse regained the ability to void urine 48 h after admission, while tail and anal tone were normal by 96 h after admission, and the horse became progressively less ataxic. He was discharged from the hospital 8 days post-admission, at which time grade 2/5 ataxia involving the pelvic limbs remained, but all other neurological deficits had resolved. The horse was returned to the barn of origin at the racetrack and was isolated in the end of the barn where other EHV-1 PCR positive horses were isolated as a group. After several weeks of hand walking, the horse resumed race training and raced on March 18, 2007, 81 days after the onset of neurological deficits.

Study population at the racetrack

Following the diagnosis of EHM in the index case, all horses under the care of the same trainer at the racetrack, as well as pony horses and outrider horses that may have come into contact with horses from that trainer's barn, were monitored over a period of 3 weeks. Whole blood samples (BD Vacutainer) and nasopharyngeal swabs (Fox Converting Swabs) were collected on several occasions over a period of 3 weeks (Table 1) from 39 adult Thoroughbred horses and 35 adult pony horses representing several breeds (Quarter Horse, American Paint Horse, Appaloosa). The Thoroughbred horses were housed in two adjacent barns, barn 71 (31 horses, including the index case) and barn 72 (8 horses) under the care of the same trainer, assistant trainer and staff of exercise riders, grooms and barn personnel. The 35 pony horses were used to accompany the Thoroughbred horses during training sessions and were kept in five different barns (barn 101, 19 horses; barn 43, 4 horses; barn 34, 4 horses; barn 25, 7 horses; barn 14, 1 horse) which were distant from barns 71 and 72.

Vaccination records were available for only 19/39 Thoroughbreds, which had been vaccinated with an inactivated EHV-1/influenza combination product (Calvenza EHV/EIV, Boehringer Ingelheim) 5–10 months

Table 1
Identification, initial location and sampling times of 40 Thoroughbred horses and 35 pony horses involved in an EHV-1 myeloencephalopathic outbreak

Identification	Use	Initial location (Barn/Stall)	Collection dates (number of horses sampled)						
			28 Dec	30 Dec	2 Jan	5 Jan	7 Jan	9 Jan	21 Jan
Horse 1	Racing horse	71/15	1	1	1	1	0	1	1
Horses 2–9	Racing horses	72/10–15,18,19	0	8	0	1	1	8	6
Horses 10–40		71/1–14,17,19–33,35	0	31	6	3	4	31	29
Horses 41–59	Pony horses	101/34,35,38–43,45,46, 48–50,52–57	0	9	10	2	0	9	0
Horses 60–63		43/49–52	0	0	4	0	0	0	0
Horses 64–67		34/1,3,36,37	0	4	0	1	0	4	0
Horses 68–74		25/1–7	0	4	3	0	0	4	0
Horse 75		14/2	0	0	1	0	0	0	0
Total			1	57	25	8	5	57	36

previously. Vaccination records were not available for the adult pony horses.

Sample collection

Disposable gloves were worn by the personnel who collected samples or handled the horses and gloves were changed between each horse, with care taken not to cross-contaminate the swabs and/or tubes. Low risk horses, i.e. those housed furthest from the index case or other PCR-positive horses, were sampled before high risk horses in order to minimize the potential for disseminating EHV-1 infection during sample collection. All samples were kept refrigerated until processed for nucleic acid extraction within 24 h of collection.

Management during the outbreak at the racetrack

The initial index case (horse 1) was stabled in barn 71, stall 15 (Fig. 1). This horse had a 3 day history of fever preceding the acute onset of neurological signs that necessitated referral to the VMTH later that same day. The horse had been training prior to the initial onset of fever and had been in direct contact with horses housed in adjacent stalls, as well as with pony horses. In addition, this horse had indirect contact with additional horses through shared barn staff, exercise riders, tack and equipment. Horse 40, stabled adjacent to the index case in barn 71, stall 14, was reported to have had a fever 1 week before horse 1 developed fever. These two horses had been stabled in the same stalls for at least 4 months and had not been vaccinated during this time. Two horses, horses 17 and 33 stabled in barn 71, stalls 27 and 7, respectively, had recently returned to the barn after competing at a racetrack in Southern California.

Following the diagnosis of the index case, barn 71 and 72 were quarantined and all horses at the racetrack were monitored daily for fever ($T > 38.5$ °C), nasal discharge and neurological signs. Four stalls in barn 71 (16–19) were initially used to isolate horses that had tested positive for EHV-1 by PCR and thus considered to pose a risk for other horses. Additional vacant stalls in the North-eastern part of the barn were also used to confine high risk horses. The quarantine was lifted 14 days after the index case was removed from the premises based on the lack of additional clinical cases. Molecular results were not considered by the regulatory authorities in the lifting of the quarantine. During the quarantine period, no additional horses in the study population developed fever or other signs compatible with EHV-1 infection.

Sample processing and nucleic acid extraction

Five milliliters of phosphate-buffered saline (PBS) were added to each swab. Thereafter, each swab was vortexed for 10 s, inverted and centrifuged at 16,000 *g* for 5 min in order to retrieve a cell pellet. After removing the swab and supernatant, each pellet was re-suspended into 400 μL of PBS prior to nucleic acid extraction. In order to minimize contamination, all pipetting steps were performed under laminar flow. Nucleic acid extraction from whole blood and NPS was performed using an automated nucleic acid extraction system (CAS-1820 X-tractor Gene, Corbett Life Science) according to the manufacturer's recommendations. For the automated extraction, 180 μL of each sample (whole blood and re-suspended nasopharyngeal cell pellet) were used.

Total RNA was purified from blood and NPS as follows: 20 μL of each freshly extracted nucleic acid sample (containing genomic DNA and total RNA) were digested with DNAse for 60 min at 37 °C to remove genomic DNA (gDNA). DNAse was inactivated at 95 °C for 5 min. Before cDNA synthesis, 1 μL of each sample was tested for gDNA background using the housekeeping gene equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a marker. Only samples negative for equine GAPDH were processed for cDNA synthesis, while positive samples were re-digested and re-tested. Complementary DNA (cDNA) from each sample was synthesized using 50 U SuperScript III (Invitrogen) in a 40 μL final volume containing 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 0.5 mM dNTPs, 40 U RNAsin, 0.5 mM dithiothreitol (DTT) and 600 ng random hexadeoxyribonucleotide (pd(N)6) primers (random hexamers; Invitrogen). The reaction was performed at 50 °C for 60 min. After inactivation at 95 °C for 5 min, the reaction volume was adjusted to 100 μL with nuclease-free water and cDNA samples were stored at −20 °C.

Real-time PCR analysis

To determine the efficiency of nucleic acid extraction we analyzed all samples for the presence of equine GAPDH gene as previously described (Pusterla et al., 2006). This target gene is routinely used in our laboratory as a housekeeping gene in order to determine quality control of samples. All samples were assayed for the presence of the *gB* gene using a previously reported real-time TaqMan PCR assay (Pusterla et al., 2006). Briefly, the real-time TaqMan PCR assay used is based on the detection of a specific 90 base-pair long product of the *gB* gene of EHV-1 (*ORF33*; GenBank accession number NC_001491; oligonucleotides: forward primer TATACTCGCTGAGGATGGAGACTTT, reverse primer TTGGGGC-AAGTTCTAGGTGGTT, probe 6FAM-ACACCTGCCCACCGCCTA-CCG). Amplification efficiency of the *gB* gene assay was calculated from the slope of a standard curve generated on 10-fold diluted EHV-1 positive DNA sample. High amplification efficiency for the target gene of 95% indicated a high analytical sensitivity. Analytical specificity was verified by sequencing TaqMan PCR products for the target gene and using positive (DNA from whole EHV-1 virus) and negative controls (EHV-1-free DNA from whole blood and nasal secretions) with each run.

Differentiation between non-neuropathogenic and neuropathogenic EHV-1 field strains was based on the $A_{2254}G$ single nucleotide polymorphism in the *DNA polymerase* gene (*ORF 30*), as previously reported (Leutenegger et al., in press). Briefly, the real-time TaqMan PCR assay used is based on the detection of a specific 54 base-pair long product of the polymerase-based EHV-1 gene using a TaqMan MGB probe specific for the

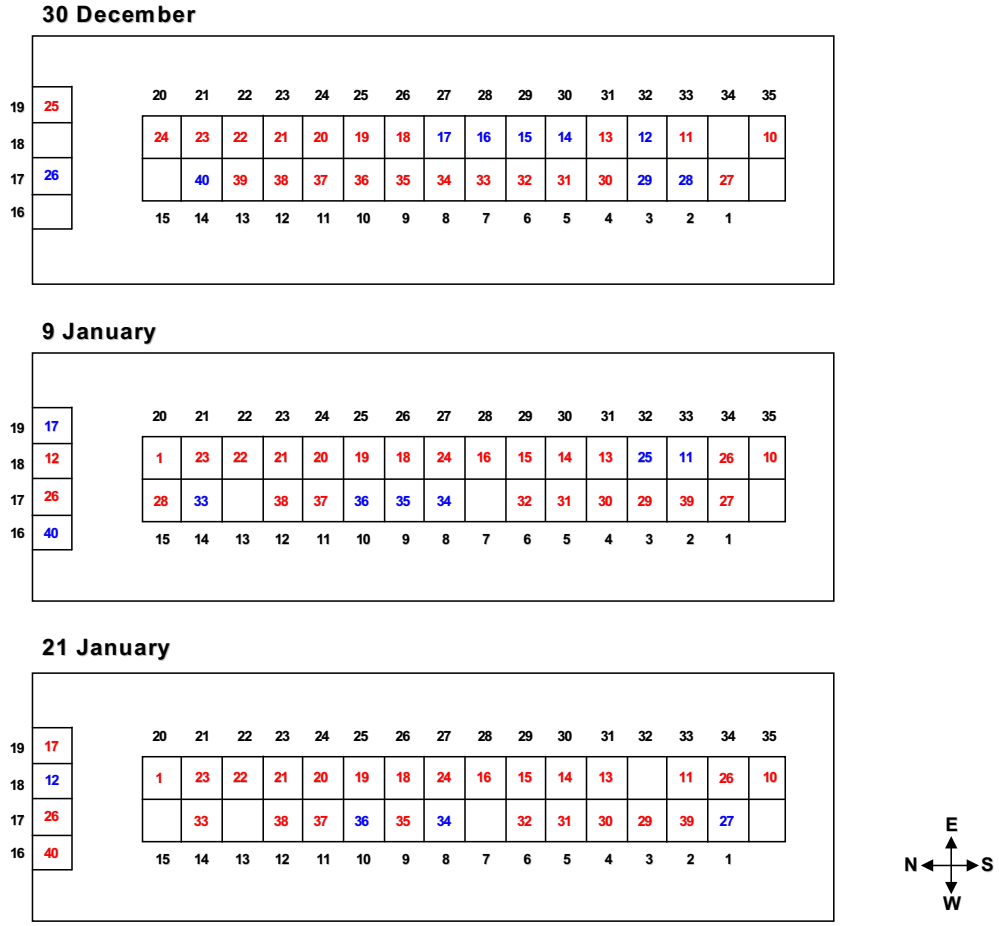

Fig. 1. A map of barn 71 showing the stall numbers (black) as well as horses that tested PCR positive (blue) or negative (red) by the collection dates. The index case was initially stabled in stall 15.

neuropathogenic EHV-1 strain (reference strain Ab4; GenBank accession number AY665713; oligonucleotides: probe 6FAM-ATCCGTCGACTACTCG, forward primer ATCTGGCCGGGCTTCAAC, reverse primer GGTCACCCACCTCGAACGT) and a TaqMan MGB probe specific for non-neuropathogenic EHV-1 strain (reference strain V592; GenBank accession number AY464052; oligonucleotides: probe VIC-ATCCGTCAACTACTCG, forward primer ATCTGGCCGGGCTTCAAC, reverse primer GGTCACCCACCTCGAACGT).

The *DNA polymerase* EHV-1 real-time TaqMan PCR assays were validated for their discriminatory ability of the single nucleotide polymorphism at nucleotide position 2254 (A–G transition) using synthetic oligonucleotides (EHV-1 neuropathogenic control: TGAAGTGGATCTGGCCGGGCTTCAACCATCCGTCGACTACTCGACGTTCGAGGTGGGTGACCAAAAGTTA; EHV-1 non-neuropathogenic control: TGAAGTGGATCTGGCCGGGCTTCAACCATCCGTCAACTACTCGACGTTCGAGGTGGGTGACCAAAAGTTA) of the *DNA polymerase* target region for both isolates. In addition, standard curves were run for both assays and the amplification efficiency calculated from the slope using the formula $E = 10^{-1/s} - 1$. High amplification efficiency for the *ORF 30* target gene of 98% indicated a high analytical sensitivity. Analytical specificity was verified by sequencing TaqMan PCR products for the target gene and using positive (synthetic oligonucleotides) and negative controls (EHV-1-free DNA from whole blood and nasal secretions) with each run. An additional real-time TaqMan PCR assay targeting the *ORF 64* gene was established and validated in order to determine the transcriptional activity of LATs of EHV-1.

The EHV-1 LATs assay is based on the detection of a specific 113 bp long product of the *ORF 64* gene of EHV-1 (GenBank accession number: AF464052; oligonucleotides: forward primer GGGTGCTGGAGGTGAGGAC, reverse primer GCGATCAGCCAGTACCACATC, probe 6FAM-GGCTGAGG). The *ORF 64* TaqMan PCR assay was validated as previously described (Leutenegger et al., 1999). Briefly, amplification efficiencies were calculated from the slopes of standard curves generated on 10-fold diluted EHV-1 positive DNA samples. High amplification efficiencies for *ORF 64* of 96% indicated a high analytical sensitivity. Analytical specificity was verified by sequencing TaqMan PCR products for the target gene and using positive (synthetic oligonucleotides) and negative controls (EHV-1-free DNA from whole blood and nasal secretions) with each run.

The amplification conditions for all the tests (EHV-1 gB gDNA, gB cDNA, ORF 30 gDNA and ORF 64 cDNA and equine GAPDH) were identical and the samples were amplified in a combined thermocycler/fluorometer (7900 HTA, Applied Biosystems) with the standard thermal cycling protocol: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The PCR reactions for each assay were composed of a commercially available mastermix (Universal TaqMan Mastermix with AmpErase UNG, Applied Biosystems), containing 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM dUTP, 0.625 U of AmpliTaq Gold per reaction, 0.25 U AmpErase UNG per reaction, 400 nM of each primer and 80 nM of the respective TaqMan probe, and 1 μL of gDNA or 5 μL of cDNA sample for a total volume of 12 μL. Final quantitation of EHV-1 DNA and RNA load for the *gB* gene was done as follows: absolute numbers of EHV-1 genomes were extrapolated to a standard curve generated with cloned EHV-1 *gB* fragments. In addition, the equine GAPDH cycle threshold values (CT values represent the fractional cycle number at which the fluorescence passes the fixed threshold) obtained in parallel to the EVH-1 TaqMan PCR were used to calculate genome equivalents

present per PCR reaction and cell number after extrapolation to a standard curve generated on cloned equine GAPDH fragments, knowing that every diploid eukaryotic cell has two copies of the equine GAPDH pseudogene. Thereafter, the results of EHV-1 gDNA for *gB* gene were expressed as copies per million cells (either peripheral blood leukocytes or nasopharyngeal cells). For absolute quantitation of the RNA load for the *gB* gene, the EHV-1 raw data was extrapolated to the respective standard curve and then combined with the cell number determined on the gDNA fraction of the same sample. Viral state was determined based on the molecular signature determined for each sample at the gDNA and cDNA level (Table 2).

Results

Index case

Genomic DNA from blood and NPS of horse 1, the clinically affected index case, were positive for the presence of both EHV-1 *gB* DNA (Fig. 2) and neuropathogenic *ORF 30* DNA and negative for the non-neuropathogenic *ORF 30* gene from the day of admission through day two and day three of hospitalization, respectively. EHV-1 viral loads in blood at the gDNA and cDNA level declined rapidly and were 10,000 fold lower than viral loads in NPS. EHV-1 viral loads in NPS at the gDNA and cDNA displayed a similar slow decline over a period of 60 h before becoming negative at 72 h of hospitalization, at which time horse 1 had received the seventh of a total of 16 oral doses of the antiviral drug valacyclovir. Horse 1 remained EHV-1 PCR negative on both blood and NPS on follow-up tests (9 and 21 January).

Table 2
Molecular signature to differentiate viral states of EHV-1 using real-time PCR

Target gene	Nucleic acid	Viral state		
		Lytic	Non-replicating	Latent
Glycoprotein B	gDNA	Yes	Yes	Yes
	cDNA	Yes	No	No
ORF 64	cDNA	No	No	Yes

EHV-1 detection, viral loads and state on 30 December

During the first collection (48 h after admission of horse 1 to the VMTH Isolation unit), 13 Thoroughbred racehorses and one pony horse tested EHV-1 PCR positive for *gB* DNA in NPS (Table 3). EHV-1 viral loads at the gDNA level in NPS of these horses ranged from 62 to 1.9×10^8 gene copies/million cells, with only six horses displaying transcriptional activity of the *gB* gene. Four clusters of viral activity were observed, one in barn 72 involving four horses in close contact with each other, and three clusters in barn 71 involving a total of nine horses. A cluster of five positive horses was found in the Eastern part of the barn 71 and two separate clusters each comprising two horses were seen in the Western part of the same barn. The two horses in one of these clusters were horse 40, the horse housed in stall 14 adjacent to stall 15 where the index case had been housed, and horse 26 housed in stall 17 directly across the aisle from stall 15.

Only four horses, all of which tested PCR positive for EHV-1 *gB* DNA in NPS, also tested PCR positive for EHV-1 *gB* DNA in blood. EHV-1 viral loads in blood ranged from 1.8×10^2 to 4.9×10^3 gene copies/million cells; however, only two of these horses displayed transcriptional activity of *gB* gene in blood. Transcriptional activity of *ORF 64* was seen in only two blood samples. The viral strain could not be determined due to lack of detectable signal in six samples (two blood and four NPS) that tested EHV-1 PCR positive for *gB* DNA. All these samples had low *gB* gene viral loads at the DNA level, ranging from

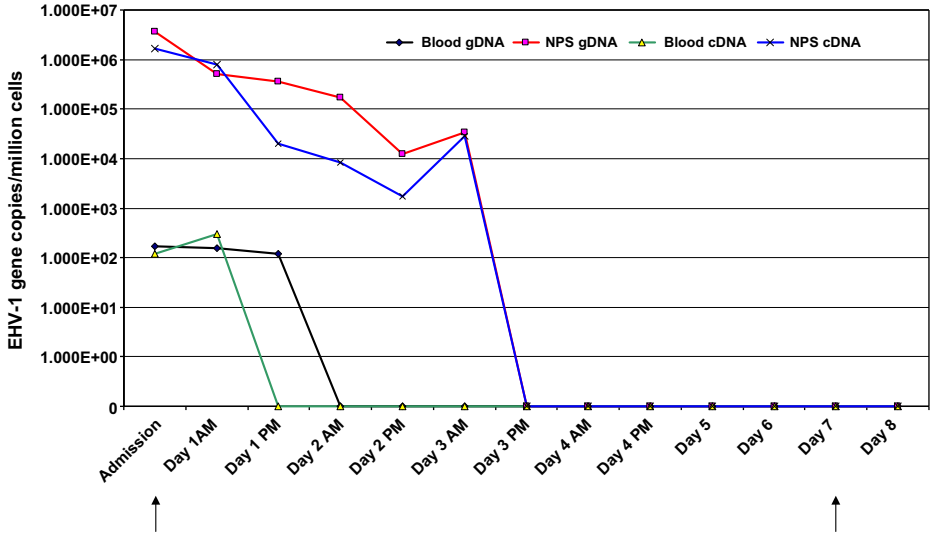

Fig. 2. *Glycoprotein B* gene loads determined by real-time PCR in blood and nasopharyngeal secretions (NPS) at the gDNA and cDNA level of horse 1 (index case) during hospitalization. The results are expressed as number of EHV-1 *gB* gene copies per million cells. The arrows represent the time frame horse 1 was treated with valacyclovir.

Table 3
Horses positive for EHV-1 by PCR performed on blood and nasopharyngeal secretions (NPS) collected from Thoroughbred horses and pony horses at the racetrack on 30 December

Horse ID	Viral load gDNA *gB* (copies/million cells)		Viral load cDNA *gB* (copies/million cells)		Transcriptional activity of LATs		Neuropathogenic strain (*ORF 30*)		Non-neuropathogenic strain (*ORF 30*)		Viral state	
	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS
4	4.9×10^{3}	9.8×10^{3}	1.6×10^{3}	1.3×10^{3}	–	–	–	–	–	Yes	Lytic	Lytic
5	–	1.7×10^{2}	–	–	–	–	–	–	–	–	–	Non-replicating
7	–	7.8×10^{3}	–	6.2×10^{2}	–	–	–	Yes	–	Yes	–	Lytic
8	–	3.6×10^{2}	–	–	–	–	–	–	–	–	–	Non-replicating
12	1.9×10^{2}	2.8×10^{6}	–	2.3×10^{5}	Yes	–	–	Yes	–	Yes	Latent	Lytic
14	–	1.0×10^{3}	–	–	–	–	–	–	–	–	–	Non-replicating
15	–	3.0×10^{2}	–	–	–	–	–	–	–	Yes		Non-replicating
16	–	1.0×10^{3}	–	8.9×10^{1}	–	–	–	–	–	Yes	–	Lytic
17	4.7×10^{3}	2.7×10^{5}	4.8×10^{4}	1.0×10^{3}	–	–	Yes	Yes	Yes	Yes	Lytic	Lytic
26	–	6.2×10^{1}	–	–	–	–	–	–	–	Yes	–	Non-replicating
28	1.8×10^{2}	1.9×10^{8}	–	2.1×10^{7}	Yes	–	–	Yes	Yes	Yes	Latent	Lytic
29	–	1.3×10^{2}	–	–	–	–	–	–	–	–	–	Non-replicating
40	–	6.4×10^{3}	–	–	–	–	–	Yes	–	–	–	Non-replicating
44	–	2.3×10^{2}	–	–	–	–	–	–	–	Yes	–	Non-replicating

1.3×10^{2} to 4.9×10^{3} copies/million cells. Dual infection with both strains (neuropathogenic and non-neuropathogenic) was detected in five samples (four NPS, one blood) collected from four horses. Five horses tested EHV-1 positive in NPS but not in blood for only the non-neuropathogenic strain, while one horse tested EHV-1 positive in NPS for only the neuropathogenic strain (Table 3). Interestingly, the only horse that tested EHV-1 positive for the neuropathogenic EHV-1 strain in NPS was horse 40, the one stabled next to the index case. Based on the viral signatures, viral state was determined to be lytic in eight samples (six NPS, two blood), non-replicating in eight NPS and latent in two blood samples (Table 3).

Following initial PCR results, available within 24 h of collection, the following horses were kept isolated in the Northern and North-eastern part of barn 71: horse 26 and 40 (in close-contact with index case); 17 and 33 (returning from racetrack in Southern California); 12 and 28 (high viral loads in NPS).

EHV-1 detection, viral loads and viral state between 2 and 7 January

From 2 until 7 January, 31 selected horses were re-tested on one or several occasions to follow viral kinetics (Table 4). Eleven horses (seven racehorses and four pony horses) were found to be PCR positive for EHV-1 *gB* DNA in NPS, while two horses also tested EHV-1 PCR positive in blood. The viral loads in NPS for *gB* gene at the gDNA level varied from 89 to 3.0×10^{7} gene copies/million cells. Only five horses showed transcriptional activity of the *gB* gene in NPS. Interestingly, horse 33, one of the two horses that had recently returned back from Southern California, had the highest viral loads at the gDNA and cDNA level. This horse was subsequently isolated as a precautionary measure.

When compared to the viral loads of the *gB* gene measured on 12/30, horse 4 located in barn 72 was the only one to show an increase in viral load, while horses 12, 16, 17 and 28, all located in barn 71, had lower viral loads with no evidence of transcriptional activity on follow-up analysis. Transcriptional activity of LATs was found in the blood and NPS of one and two horses, respectively. Viral strain using the ORF 30 assays could not be determined in four samples (one blood, three NPS) from three horses. Two horses (17, 67) and one time point and one horse at two time points (horse 33) showed dual infection with both strains in NPS only, while nine were positive for only non-neuropathogenic stains (one blood, eight NPS) (Table 4).

Based on the viral signatures, viral state was determined to be lytic in five NPS samples, non-replicating in one blood sample and eight NPS, and latent in one blood samples and two NPS (Table 4). During this time period no additional horses were moved to the isolation area of barn 71. Horse 1 was placed in barn 71, stall 20 when it returned from the VMTH on 5 January, 2007, while the horse initially kept in this stall (horse 24) was moved to stall 27. Stall 20 was included in the isolation area even though horse 1 was considered to be non-infectious based on repeated negative EHV-1 PCR results on blood and NPS.

EHV-1 detection, viral loads and state on 9 January

Ten days after the first collection, 12/57 horses (21%; 10/40 racing horses; 2/17 pony horses) tested PCR positive for EHV-1 *gB* DNA (Table 5). A new cluster of three EHV-1 positive horses (34–36) was observed in the Western part of barn 71. These horses were stabled adjacent to stall seven, where horse 33 had been housed when it returned from racing in Southern California. This horse had been moved to the isolation area 7 days previously (2 January, 2007). One horse was EHV-1 positive only in blood, two horses were

Table 4
Horses positive for EHV-1 by PCR performed on blood and nasopharyngeal secretions (NPS) collected from Thoroughbred horses and pony horses at the racetrack from 2 to 7 January

Horse ID (date)	Viral load gDNA *gB* (copies/million cells)		Viral load cDNA *gB* (copies/million cells)		Transcriptional activity of LATs		Neuropathogenic strain (*ORF 30*)		Non-neuropathogenic strain (*ORF 30*)		Viral state	
	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS
4 (1/2)	–	1.6×10^5	–	2.9×10^4	–	–	–	–	–	Yes	–	Lytic
12 (1/2)	–	3.5×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating
16 (1/2)	–	8.9×10^1	–	–	–	–	–	–	–	–	–	Non-replicating
17 (1/2)	–	1.0×10^3	–	–	–	Yes	–	Yes	–	Yes	–	Latent
17 (1/5)	–	5.9×10^2	–	–	–	–	–	–	–	Yes	–	Non-replicating
19 (1/2)	–	2.5×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating
28 (1/2)	–	4.0×10^2	–	–	–	–	–	–	–	Yes	–	Non-replicating
28 (1/5)	9.9×10^1	3.9×10^2	–	–	–	–	–	–	–	–	Non-replicating	Non-replicating
33 (1/2	1.4×10^2	6.1×10^5	–	1.5×10^3	Yes	–	–	–	Yes	–	Latent	Lytic
33 (1/5)	–	2.9×10^6	–	1.9×10^3	–	–	–	Yes	–	Yes	–	Lytic
33 (1/7)	–	3.0×10^7	–	3.8×10^5	–	–	–	Yes	–	Yes	–	Lytic
67 (1/2)	–	9.8×10^5	–	4.9×10^4	–	–	–	Yes	–	Yes	–	Lytic
69 (1/2)	–	2.5×10^3	–	–	–	Yes	–	–	–	Yes	–	Latent
71 (1/2)	–	2.4×10^2	–	–	–	–	–	–	–	Yes	–	Non-replicating
74 (1/2)	–	2.5×10^2	–	–	–	–	–	–	–	Yes	–	Non-replicating

Table 5
Horses positive for EHV-1 by PCR performed on blood and nasopharyngeal secretions (NPS) collected from Thoroughbred horses and pony horses at the racetrack on 9 January

Horse ID	Viral load gDNA *gB* (copies/million cells)		Viral load cDNA *gB* (copies/million cells)		Transcriptional activity of LATs		Neuropathogenic strain (*ORF 30*)		Non-neuropathogenic strain (*ORF 30*)		Viral state	
	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS
4	–	1.6×10^3	–	–	–	–	–	–	–	–	–	Non-replicating
5	–	9.4×10^2	–	–	–	–	–	–	–	Yes		Non-replicating
11	–	2.7×10^3	–	–	–	Yes	–	–	–	Yes	–	Latent
17	–	2.3×10^2	–	–	–	–	–	–	–	Yes	–	Non-replicating
25	–	3.7×10^3	–	–	–	Yes	–	–	–	Yes	–	Latent
33	–	3.4×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating
34	2.7×10^2	3.4×10^5	–	3.5×10^3	Yes	–	–	Yes	–	Yes	Latent	Lytic
35	7.1×10^1	5.1×10^6	–	3.4×10^4	–	–	–	Yes	–	Yes	Non-replicating	Lytic
36	–	2.5×10^6	–	2.7×10^5	–	–	–	Yes	–	Yes	–	Lytic
40	4.6×10^2	–	–	–	Yes	–	–	–	–	–	Latent	–
67	–	7.8×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating
70	–	5.6×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating

EHV-1 positive in blood and NPS and nine horses tested EHV-1 positive only in NPS. Viral loads at the gDNA level ranged from 71 to 4.6×10^2 gene copies/million cells and from 2.3×10^2 to 5.1×10^6 gene copies/million cells in blood and NPS, respectively.

The three new EHV-1 positive horses (34, 35, and 36) housed in stalls 8, 9, and 10 in the Western part of barn 71 were the only ones displaying transcriptional activity of the *gB* gene in NPS. Transcriptional activity of *ORF 64* was seen in two blood and two NPS samples from four horses. Viral strain could not be determined due to lack of detectable signal in four samples (three bloods and one NPS) that were positive for EHV-1 *gB* DNA. Three horses tested EHV-1 positive for both neuropathogenic and non-neuropathogenic strains while seven horses tested EHV-1 positive for only the non-neuropathogenic strain (Table 5). Based on the viral signatures, viral state was determined to be non-replicating in seven samples (six NPS, one blood), latent in four samples (two NPS, two blood) and lytic in three NPS (Table 5).

EHV-1 detection, viral loads and state on 21 January

On the last collection date, 22 days after the first visit to the racetrack, 5/36 racehorses (14%) tested EHV-1 PCR positive (Table 6). With the exception of horse 27, all other horses had tested EHV-1 PCR positive on previous occasions. Only NPS were positive for EHV-1 *gB* DNA (viral loads ranged from 2.3×10^2 to 1.6×10^5 gene copies/million cells), with transcriptional activity of the *gB* gene reported for two horses. No transcriptional activity of *ORF 64* was seen in any sample. Viral strain could not be determined in

Table 6
Horses positive for EHV-1 by PCR performed on blood and nasopharyngeal secretions (NPS) collected from Thoroughbred horses and pony horses at the racetrack on 21 January

Horse ID	Viral load gDNA *gB* (copies/million cells)		Viral load cDNA *gB* (copies/million cells)		Transcriptional activity of LATs		Neuropathogenic strain (*ORF 30*)		Non-neuropathogenic strain (*ORF 30*)		Viral state	
	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS	Blood	NPS
5	–	2.2×10^3	–	–	–	–	–	–	–	Yes	–	Non-replicating
12	–	3.6×10^3	–	–	–	–	–	–	–	Yes		Non-replicating
27	–	1.6×10^5	–	1.8×10^3	–	–	–	Yes	–	Yes	–	Lytic
34	–	2.3×10^2	–	–	–	–	–	–	–	–	–	Non-replicating
36	–	1.2×10^5	–	5.8×10^3	–	–	–	Yes	–	Yes	–	Lytic

one sample, while the remaining samples tested EHV-1 positive for only the non-neuropathogenic strain (two NPS) or for both neuropathogenic and non-neuropathogenic strains (two NPS) (Table 6). Based on the viral signatures, viral state was determined to be non-replicating in three NPS and lytic in two NPS, neither of which were the neuropathogenic strain (Table 6).

When evaluating the EHV-1 status of racehorses in relation to the prior documented use of vaccines, there was no apparent difference between vaccinated and unvaccinated horses and the detection of EHV-1 *gB* DNA (7 vaccinated horses tested EHV-1 positive, 12 vaccinated horses tested EHV-1 negative, 11 unvaccinated horses (including the index case) tested EHV-1 positive and 10 unvaccinated horses remained EHV-1 negative throughout the study).

Discussion

This paper describes viral loads, strain and state of EHV-1 measured by real-time PCR in the blood and NPS of adult horses following natural exposure. It is widely (but erroneously) assumed that molecular detection of EHV-1 in blood samples and NPS indicates viremia from active infection and shedding of infectious virus, respectively. However, one must bear in mind that PCR assays in routine use in diagnostic laboratories for molecular diagnosis of viral infection do not allow documentation of the biological state of the virus (i.e. replicating, non-replicating, or latent), nor are results typically reported in a quantitative manner that allows assessment of viral loads. Practicing veterinarians and regulatory officials who submit samples and receive positive PCR test results are typically unaware of the complexities involved in test interpretation and could decide to quarantine equine facilities or cancel competitions by misinterpreting test results. These possibilities support the need to reach a consensus on use and interpretation of molecular diagnostic techniques in the evaluation of suspected field cases of EHV-1 infection and disease. Molecular characterization of EHV-1 in field samples was achieved in this study by using absolute quantification and targeting viral DNA and transcriptional activity of the target genes.

Using absolute quantification of viral loads in blood and NPS, we demonstrated low-level viremia (i.e. presence of detectable EHV-1 target genomes in blood) accompanied by high-level viral shedding (i.e. presence of detectable EHV-1 target genomes in NPS) in the index case at the onset of neurological signs. Neurological deficits associated with EHV-1 infection commonly follow the development of fever and peak viremia by a few days (Slater, 2007). Anecdotally, many veterinarians mistakenly believe that horses with neurological manifestations of EHV-1 infection are no longer shedding virus.

The results of this investigation and another study performed in our laboratory (unpublished data) clearly demonstrate that horses with EHM shed high loads of replicating EHV-1 in NPS and therefore pose a substantial risk to other horses. From a biosecurity standpoint, horses affected with EHM should be isolated and confined away from other horses in order to decrease risk of exposure of susceptible horses. Although the risk of transmission of EHV-1 from neurologically affected horses has not been thoroughly evaluated, indirect spread by fomites and personnel is the more likely reason for the rapid spread of infection during outbreaks of EHM. The highly infectious nature of such horses has been well demonstrated during recent outbreaks at racetracks, riding schools and veterinary hospitals (Kohn et al., 2006; Henninger et al., 2007).

High viral loads and transcriptional activity for the *gB* gene were recorded for the first 60 h of hospitalization in the index case, followed by a rapid decline within the next 12 h, while lytic infection in blood was recorded only during the first 12 h of hospitalization. It appears likely that the use of the antiviral drug valacyclovir contributed to the rapid decline in transcriptional activity of the *gB* gene in blood and NPS. This observation is supported by the fact that viral loads of EHV-1 in the nasal secretions of experimentally infected horses display a more gradual decline over time (Hussey et al., 2006). Preliminary pharmacokinetic studies have shown greater bioavailability of the nucleoside analogue valacyclovir when compared to acyclovir (Bentz et al., 2007), although further experimental in vivo studies will be needed to fully document the efficacy of valacyclovir to reduce viral loads and shedding, and promote clinical recovery.

The prevalence of EHV-1 infection in NPS determined by PCR slowly decreased over the 22 day study period from 25% to 14%. Interestingly, the high prevalence

encountered initially does not appear to have been attributable to the rapid spread of a neuropathogenic strain, since only two horses (index case, horse 1, and stall neighbor horse 40) tested PCR positive for only the neuropathogenic strain. It is likely that at the time the index case was first recognized, clusters of silent EHV-1 spread appeared in several areas of barns 71 and 72. Despite molecular characterization of all positive PCR results, the source of infection for the PCR positive horses could not be determined definitively.

Interestingly, two horses (17 and 33) had recently returned after racing at a track located in Southern California. Although no clinical EHM cases were reported from that racetrack, it is likely that these two horses either were exposed to a sub-clinical shedder or experienced a reactivation of EHV-1 from a latent state, or a combination of the two, during transportation or while housed in another trainer's barn while at the Southern California track. The two horses developed lytic infections, characterized by viremia and viral shedding that persisted for up to 10 days. Moving these two horses to the isolation part of the barn when positive PCR results were reported did not prevent exposure and silent infection in horses located in adjacent stalls in barn 71 (horses 11, 12, 14, 15, 16, 19, 25, 27, 28, 29, 34, 35, 36), as exemplified by additional horses displaying viremia and viral shedding. Additional silent cycles of spread of EHV-1 appeared to occur in barn 72 involving three sub-clinically infected horses (4, 5, 7) and in barn 25 between several sub-clinical pony horses (69, 70, 71, 74).

Differentiation of EHV-1 strains proved to be useful for epidemiological purposes studying this outbreak investigation. However, it is important to remember that the ORF 30 assays used to differentiate between neuropathogenic and non-neuropathogenic strains are not 100% specific. Approximately 86% of the EHV-1 isolates from horses with EHM carry the neuropathogenic marker, while 94% of isolates from non-neurologic EHV-1 disease syndromes lack the neuropathogenic marker. In other words, 14% of EHV-1 isolates from EHM cases contain the wild type, non-mutated nucleotide, while 6% of isolates from non-neurologic EHV-1 infected horses contain the mutated nucleotide (Nugent et al., 2006). Further, our results indicate that the PCR assay targeting the *gB* gene was associated with a higher detection rate when compared to the assays targeting the *ORF 30* gene. Fifteen samples (six blood and nine NPS) with positive PCR results for the *gB* gene all tested negative in the assay based on the *ORF 30* gene. Typically, those samples with low viral loads (<500 *gB* gene copies/million cells) were the ones that tested negative for the *ORF 30* gene. Although the *gB* gene PCR assay appears to be diagnostically more sensitive in detecting low viral loads in samples from field cases, the discrepancy in results for the 15 samples could also be attributed to the presence of dual strains in numbers too low to be detected with the corresponding assays, to the dilutional effect of purified target DNA or to the small amount of DNA used for each PCR reaction. Additionally, we could not rule out that additional mutations were present on the *polymerase* gene of specific isolates, making them undetectable using the present assays.

We also observed that the viral loads in horses that were dually infected with both strains were generally higher (range 180–1.9×10^8 gene copies/million cells) than the viral loads in horses with only non-neuropathogenic strains (62–1.6×10^5 gene copies/million cells). However, no conclusions can be drawn from these findings because horses previously determined to be viremic and/or shedding virus in NPS were more likely to be re-tested at subsequent sampling times. Interestingly, the main EHV-1 strains found in blood and nasal secretions from the study horses were either non-neuropathogenic or mixed. This is in disagreement with a recent study (Allen, 2007) reporting mainly on the detection of neuropathogenic EHV-1 strains in horses known to have been exposed to EHV-1 during large myeloencephalopathy epizootics. The difference in the prevalence of EHV-1 strains between the two studies is likely to be associated with the different horse populations tested and the detection of additional silent cycles of spread of EHV-1 in sub-clinical horses with no direct exposure to the index case. To the best of our knowledge, this study is the first report of detection of mixed EHV-1 strains in sub-clinical horses.

The use of molecular signatures which targeted several genes at the gDNA and cDNA level allowed characterization of the viral state in each of the EHV-1 PCR positive samples. The molecular approach used in this study represents a crude measure of very complex biological processes that are in part still poorly understood. We must also consider that during an ongoing EHV-1 infection, the viral state rarely satisfies the 'all or none' rule, but rather progressively transitions from one state to the next. The lytic or replicating state is easy to assess using molecular techniques based on the detection of transcripts from essential genes, such as the *gB* gene, that are absolutely required for virus replication. Detection of virus in the lytic state is a strong indicator of active infection and potential for viral transmission, even when it only occurs in a silent sub-clinical cycle.

Lytic infection was characterized by the presence of transcriptional activity of the *gB* gene, and, as expected, was associated with high viral loads at the gDNA level (range 1×10^3 to 1.9×10^8 copies/million cells), while non-replicating virus was routinely seen with low viral loads (64–7.8×10^3 copies/million cells). However, the detection of EHV-1 *gB* DNA without detection of transcriptional activity of the same gene may have other possible biological (i.e. abortive EHV-1 infection without the expression of late genes, early re-activation from latency) and technical (i.e. differences in analytical sensitivity of the assays, sample processing) explanations.

To help refine a non-lytic state, we also tested the samples for markers of functional latency, such as EHV-1 LATs. Biologically, latency is characterized by blockage of the transcription and translation cascade of all gene clas-

ses, with the exception of limited transcription in the region antisense to the immediate early gene (Slater, 2007). This results in expression of the EHV-1 LATs, as shown in the present study. We believe this is the first report of molecular characterization of latency in samples collected during a field outbreak of EHV-1 infection.

As expected, viral loads in blood samples (range 140–460 gene copies/million cells) and NPS (1×10^3–3.7×10^3 gene copies/million cells) from latently infected horses were low. However, due to the inherent sensitivity issues associated with molecular assays, the absence of detectable LATs could also represent latent virus with no, or undetectable, expression of LATs. The molecular approach used in this study was time consuming and most likely is not applicable to routine diagnostic testing, although, as is the case with assessment of viral load, determination of the viral state allowed indirect assessment of the risk of exposure of susceptible horses. In future, the use of viral load threshold may prove to be useful diagnostically for differentiating between lytic and non-replicating or latent virus.

Conclusions

The present study showed that the viral load, strain and state can be determined using real-time PCR on field samples collected during an EHV-1 outbreak. This molecular approach can help assess the risk of exposure to other horses and permit determination of viral kinetics in infected horses. The results of the study support the need for development of a consensus on the use and interpretation of molecular diagnostic techniques in the diagnostic evaluation of field cases of suspected EHV-1 infection, since this information may influence the risk of exposure to other horses and may help practitioners and regulatory officials make appropriate decisions regarding the management of horses that test positive on PCR.

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References

Allen, G.P., 2007. Development of a real-time polymerase chain assay for rapid diagnosis of neuropathogenic strains of equine herpesvirus-1. Journal of Veterinary Diagnostic Investigation 19, 69–72.

Allen, G.P., Breathnach, C.C., 2006. Quantification by real-time PCR of the magnitude and duration of leukocyte-associated viremia in horses infected with neuropathogenic vs non-neuropathogenic strains of EHV-1. Equine Veterinary Journal 38, 252–257.

Bentz, B.G., Maxwell, L.K., Bourne, D.W.A., Erkert, R.S., 2007. Pharmacokinetics of valacyclovir in the adult horse. In: Proceedings of the 25th Annual Meeting of the American College of Veterinary Internal Medicine, Seattle, WA, USA, p. 601.

Borchers, K., Slater, J., 1993. A nested PCR for the detection and differentiation of EHV-1 and EHV-4. Journal of Virological Methods 45, 331–336.

Edington, N., Bridges, C.G., Huckle, A., 1985. Experimental reactivation of equid herpesvirus 1 (EHV 1) following the administration of corticosteroids. Equine Veterinary Journal 17, 369–372.

Henninger, R.W., Reed, S.M., Saville, W.J., Allen, G.A., Hass, G.F., Kohn, C.W., Sofaly, C., 2007. Outbreak of neurologic disease caused by equine herpesvirus-1 at a university equestrian center. Journal of Veterinary Internal Medicine 21, 157–165.

Hussey, S.B., Clark, R., Lunn, K.F., Breathnach, C., Soboll, G., Whalley, J.M., Lunn, D.P., 2006. Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction. Journal of Veterinary Diagnostic Investigation 18, 335–342.

Kohn, C.W., Reed, S.M., Sofaly, C.D., Henninger, R.W., Saville, W.J., Allen, G.P., Premanadan, C., 2006. Transmission of EHV-1 in horses with EHV-1 myeloencephalopathy: Implications for biosecurity and review. Clinical Techniques in Equine Practice 5, 60–66.

Leutenegger, C.M., von Rechenberg, B., Huder, J.B., Zlinsky, K., Mislin, C., Akens, M.K., Lutz, H., 1999. Quantitative real-time PCR for equine cytokine mRNA in nondecalcified bone tissue embedded in methyl methacrylate. Calcified Tissue International 65, 378–383.

Leutenegger, C.M., Madigan, J.E., Mapes, S., Thao, M., Estrada, M., Pusterla, N., in press. Detection of equine herpesvirus type 1 neuropathogenic strains using real-time polymerase chain reaction in neural tissue of horses with myeloencephalopathy. Veterinary Record.

Nugent, J., Birch-Machin, I., Smith, K.C., Mumford, J.A., Swann, Z., Newton, J.R., Bowden, R.J., Allen, G.P., Davis-Poynter, N., 2006. Analysis of equine herpesvirus type 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus non-neuropathogenic disease outbreaks. Journal of Virology 80, 4047–4060.

Pusterla, N., Leutenegger, C.M., Wilson, W.D., Watson, J.L., Ferraro, G.L., Madigan, J.E., 2005. Equine herpesvirus-4 kinetics in peripheral blood leukocytes and nasopharyngeal secretions in foals using quantitative real-time TaqMan PCR. Journal of Veterinary Diagnostic Investigation 17, 578–581.

Pusterla, N., Wilson, W.D., Conrad, P.A., Barr, B.C., Ferraro, G.L., Daft, B.M., Leutenegger, C.M., 2006. Cytokine gene signatures in neural tissue of horses with equine protozoal myeloencephalitis or equine herpes type 1 myeloencephalopathy. Veterinary Record 159, 341–346.

Sharma, P.C., Cullinane, A.A., Onions, D.E., Nicolson, L., 1992. Diagnosis of equid herpesviruses -1 and -4 by polymerase chain reaction. Equine Veterinary Journal 24, 20–25.

Slater, J., 2007. Equine Herpesviruses. In: Sellon, D., Long, M. (Eds.), Equine Infectious Diseases. Saunders Elsevier, St. Louis, MO, USA, pp. 134–153.

Van Maanen, C., 2002. Equine herpesvirus 1 and 4 infections: an update. Veterinary Quarterly 24, 58–78.