Comparison of the Diagnostic Sensitivity of Nasopharyngeal and Nasal Swabs and Use of Viral Loads for the Molecular Diagnosis of Equine Herpesvirus-1 Infection

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The nasopharyngeal swabbing technique for the molecular detection of equine herpesvirus-1 (EHV-1) is not well tolerated in horses; nasal swabbing represents a viable alternative. Nasal swabs seem to be diagnostically more sensitive than nasopharyngeal swabs for the detection of low EHV-1 loads in naturally exposed horses. With regard to EHV-1 viral-load signatures in blood and nasal secretions, differences between clinically affected and subclinically affected horses and between those in the febrile and neurological stages of the disease are present on nasal swabs. The finding of high viral loads in the nasal secretions of neurological horses confirms their importance as a potential source of contagion for other horses and highlights the need for imposition of strict biosecurity measures when faced with horses with suspected or confirmed neurological EHV-1 infection. Our results also support the need for development of a consensus on the use and interpretation of molecular diagnostic techniques in the evaluation of field cases of suspected EHV-1 infection. Authors’ address: Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616; e-mail: npusterla@ucdavis.edu. © 2007 AAEP.

1. Introduction

Equine herpesvirus-1 (EHV-1) is a major equine pathogen that is responsible for well-documented syndromes of respiratory disease, abortion, neonatal foal death, and myeloencephalopathy.1 Recent outbreaks at riding schools, racetracks, and veterinary hospitals throughout North America and Europe have highlighted the importance of early detection of EHV-1. Samples routinely collected in the clinical setting to document viremia or shedding of EHV-1 are whole blood and secretions from the upper respiratory tract, respectively. Although the collection of whole blood rarely poses a problem in less compliant patients, horses frequently resist collection of nasopharyngeal secretions using long swabs. Therefore, equine practitioners commonly restrict the collection of secretions to the rostral portion of the upper respiratory tract by using short nasal swabs. Unfortunately, the diagnostic sensitivity of nasal swabs in the molecular detection of EHV-1 has, to the knowledge of the authors, not been reported.

The traditional approach of virus isolation has been supplanted in recent years by more sensitive
and rapid molecular assays; however, the increasing application of sensitive molecular diagnostic techniques for detection of pathogens in practice settings has presented new dilemmas with regard to how test results are interpreted and used by both equine practitioners and regulatory veterinarians. Because routine polymerase chain reaction (PCR) assays targeting various genes of EHV-1 are unable to differentiate between replicating (lytic) and non-replicating virus, positive PCR test results should be interpreted only in the context of clinical signs shown by infected horses. The original PCR assays were not quantitative and were, therefore, incapable of determining the amount of viral DNA present in specific samples.\(^2,3\) In recent years, novel molecular platforms, such as real-time PCR, have enabled the study of viral kinetics of EHV-1 in respiratory secretions and blood samples after experimental infection.\(^4,5\) Reporting of EHV-1 viral loads on samples submitted by veterinarians from field cases would enable practitioners to determine disease stage or response to antiviral therapy; however, this is not routinely practiced in the molecular diagnostic setting.

The objective of this study was two-fold: to compare the diagnostic sensitivity of nasal swabs with the traditionally recommended nasopharyngeal swabs for the molecular detection of EHV-1 and to determine EHV-1 viral loads by real-time PCR in blood and nasal secretions collected from clinically affected and subclinically infected adult horses.

2. Materials and Methods

Study Population and Nasopharyngeal and Nasal Swab Collection and Processing

The samples were collected during an outbreak of myeloencephalopathic EHV-1 at a Thoroughbred racetrack in northern California during December 2006 and January 2007. As part of the infectious-disease control program, a number of EHV-1 index cases and potentially exposed horses were surveyed over a period of 3 wk after diagnosis of EHV-1 myeloencephalopathy in a 3-yr-old Thoroughbred gelding. Dual samples (nasopharyngeal and nasal swabs) were collected from 119 adult Thoroughbred horses and 27 adult ponies representing several breeds (Quarter Horse, American Paint Horse, and Appaloosa). None of the 146 horses were febrile or displaying neurological signs at the time of sample collection. Nasopharyngeal swabs with rayon tips\(^a\) were advanced rapidly through the ventral meatus of the right nasal passage to the pharynx and gently rotated for 10 s before withdrawal. The same type of swab with the handle cut down to 10 cm in length was used to swab the ventral aspect of the opposite (left) nasal passage. The nasal swab was advanced 10 cm into the ventral meatus and gently rotated for 10 s before withdrawal. The swabs were placed in sterile pre-labeled conical centrifuge tubes. Disposable gloves were worn by the operator, and care was taken not to cross-contaminate the swabs and/or tubes. All samples were kept refrigerated until processed for DNA extraction within 24 h of collection. Five milliliters of phosphated-buffer saline (PBS) was added to the conical tube containing each swab. Each swab was then vortexed for 10 s, inverted, and spun down at 16,000 \(\times g\) for 5 min to retrieve a cell pellet. After removing the swab and supernatant, each pellet was resuspended into 400 \(\mu l\) of PBS solution before nucleic acid extraction. To minimize contamination, all pipetting steps were performed under laminar flow. Nucleic-acid extraction was performed using an automated nucleic-acid extraction system\(^b\) according to the manufacturer’s recommendations.

Study Population, Sample Collection, and Processing to Determine Viral Loads

The study population was comprised of 27 adult horses with clinical signs consistent with EHV-1 infection. These horses were presented either to primary care veterinarians or to the Veterinary Medical Teaching Hospital at the University of California at Davis because of acute onset of fever (fever group = 12 horses) or neurological signs (neurological group = 15 horses). These horses were involved in several confirmed EHV-1 outbreaks that occurred between June 2004 and January 2007 throughout California. An additional 41 adult Thoroughbred horses residing at a racetrack in northern California were included in the subclinically infected group. These horses were part of a surveillance study to determine the prevalence of EHV-1 in a large population of racing horses. These study horses were selected based on the molecular detection of EHV-1 in blood samples or nasal secretions and the absence of fever (temperature \(>38.5^\circ C\)), nasal discharge, cough, and neurological signs. Only horses with documented absence of clinical signs 7 days before and 7 days after sample collection were included in the study. Horses with clinical disease did not fall into these criteria. Whole anticoagulated blood samples and nasal swabs\(^a\) were available from each horse for molecular testing. DNA from blood samples and nasal swabs was extracted using a commercial DNA kit\(^c\) according to the manufacturer.

Real-Time PCR Detection

All samples were assayed for the presence of the equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and the glycoprotein B (gB) gene of EHV-1 using previously reported real-time TaqMan PCR assays.\(^3\) The efficiency of nucleic-acid extraction between the nasopharyngeal and nasal swabs was determined by analyzing the samples for the presence of equine GAPDH, a housekeeping gene routinely used in our laboratory to determine quality control of samples. All samples were amplified in a combined thermocycler/fluorometer\(^d\) 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 results were expressed as CT (cycle threshold) values, which represent the fractional cycle number at which the fluorescence passes the fixed threshold (i.e., a sample is considered positive for the specific target gene). Absolute quantitation of EHV-1 target molecules was performed using standard curves for EHV-1 and equine GAPDH and expressed as EHV-1 gene copies per one million cells (peripheral blood, nasopharyngeal, or nasal epithelial cells) as previously reported.7

Using the Mann-Whitney Test, statistical differences (p < 0.05) in extraction efficiency of the equine GAPDH gene and EHV-1 between the different sample types and viral loads was determined in the horse groups.

3. Results

Comparison Between Nasopharyngeal and Nasal Swabs

DNA was successfully extracted from all 146 nasopharyngeal and nasal swabs. The average CT value and SD of equine GAPDH for nasopharyngeal and nasal swabs was 21.93 ± 2.08 and 22.81 ± 2.43, respectively. The difference in CT values for equine GAPDH between the two sample types was not statistically significant (p = 0.106). EHV-1 was detected in 24 nasopharyngeal swabs and 28 nasal swabs. Nineteen animals tested EHV-1 positive in both samples (Table 1), and average CT value and SD of the gB gene for nasopharyngeal and nasal swabs was 33.66 ± 5.81 and 32.87 ± 4.78, respectively. The difference in CT values for the gB gene between the two sample types from these 19 horses was not statistically significant (p = 0.389). The absolute EHV-1 load ranged from 210 to 1.9 × 10^8 (median = 9439) and from 131 to 1.9 × 10^6 (median = 6464) copies/million cells for nasopharyngeal and nasal swabs, respectively (p > 0.05). Five horses tested EHV-1 positive only on nasopharyngeal swabs (37.45 ± 2.46 CT value), whereas 9 horses tested EHV-1 positive only on nasal swabs (35.81 ± 4.29 CT value; p > 0.05). The median absolute viral load in the two sample types was similar: 2555 and 2400 copies/million cells for nasopharyngeal and nasal swabs, respectively. When the combined (nasopharyngeal and nasal) CT values of the gB gene for the 19 dual-positive samples were compared with the CT values of the 14 single-positive nasopharyngeal and nasal samples, the difference was statistically significant (p = 0.01). Significant difference (p = 0.006) was also determined when absolute EHV-1 load was compared with the 19 dual-positive samples (median load = 7952 EHV-1 copies/million cells) and the 14 single samples (median load = 511 EHV-1 copies/million cells).

Viral Loads in Field Specimens

EHV-1 was detected in samples of both blood and nasal secretions from all febrile horse (Table 2). Viral loads were significantly higher (p = 0.003) in blood samples (mean ± SD = 1.7 × 10^3 ± 9.7 × 10^3 gene copies/million cells) than in nasal secretions (2.8 × 10^3 ± 2.5 × 10^3 gene copies/million cells). Only 9 of the 15 neurological horses tested PCR positive in blood samples, whereas EHV-1 was detected in the nasal secretions of all 15 horses. When comparing only the samples from the nine neurological horses with dual-positive results, viral loads in nasal secretions (1.4 × 10^6 ± 1.2 × 10^6 gene copies/million cells) were significantly higher (p = 0.004) than in blood samples (270 ± 42 gene copies/million cells). Only five subclinically infected horses had positive PCR results in blood, whereas 40 of the 41 subclinical horses tested PCR positive in nasal secretions. When viral loads in blood were compared between the groups, febrile horses had significantly higher viral loads (p = 0.001) than neurological and subclinical horses. In contrast, viral loads in nasal secretions of neurological horses were significantly higher than those of febrile horses (p = 0.001) and subclinically infected horses (p = 0.001). No significant difference was found in the nasal viral

<table>
<thead>
<tr>
<th>Group (no horses)</th>
<th>Sample Type</th>
<th>PCR Positive Result (%)</th>
<th>Viral Loads (gene copies/million cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Febrile (12)</td>
<td>Blood</td>
<td>12 (100%)</td>
<td>2.3 × 10^3</td>
</tr>
<tr>
<td></td>
<td>Nasal secretions</td>
<td>12 (100%)</td>
<td>2.5 × 10^2</td>
</tr>
<tr>
<td>Neurological (15)</td>
<td>Blood</td>
<td>9 (60%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nasal secretions</td>
<td>15 (100%)</td>
<td>1.3 × 10^4</td>
</tr>
<tr>
<td>Subclinical (41)</td>
<td>Blood</td>
<td>5 (12%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nasal secretions</td>
<td>40 (97%)</td>
<td>0</td>
</tr>
</tbody>
</table>
loads between febrile and subclinical horses (p > 0.05).

4. Discussion

Although the collection of nasal swabs for the molecular detection of EHV-1 is routinely performed by equine practitioners because of better patient tolerance and client acceptance, its diagnostic sensitivity, when compared with the standard nasopharyngeal swabbing, has not been determined. The present study shows similar recovery and quality of DNA between the nasopharyngeal and nasal swabs based on similar CT values for the equine GAPDH gene, which is a prerequisite for a sensitive and accurate method of detection of the target gene (i.e., EHV-1 gB gene). Both nasopharyngeal and nasal swabs yielded similar nucleic-acid recovery in this study; however, the authors speculate that nasal swabs are more likely to contain target DNA, because mucus and exfoliated cells from the upper airways are drained through the ventral meatus by gravity and mucociliary clearance. Viral state, viral load, and disease stage will likely influence the molecular detection of EHV-1. In 19 study horses, EHV-1 was detected concurrently in nasopharyngeal and nasal swabs with no statistical difference in CT values. An additional 14 horses tested PCR positive in either one of the sample types. Almost twice as many nasal swabs (9 horses) tested PCR positive alone compared with nasopharyngeal swabs (5 horses). The CT values and absolute viral load for the gB gene in these 14 horses was significantly lower than in the 19 horses that tested PCR positive in both nasopharyngeal and nasal swabs. Although nasal swabs seem to be diagnostically more sensitive in the detection of low EHV-1 viral load, the discrepancy between the 14 horses can also be linked to the dilution effect of extracted target DNA (total extracted DNA resuspended in 80 µl of sterile water) or to the small amount of DNA (1 µl) used for each PCR reaction. The use of triplicates for each sample and/or a larger volume of DNA (up to 5 µl) per reaction may have increased the incidence of EHV-1 positive results; however, for diagnostic purposes, such an approach is unrealistic. Furthermore, based on work performed on EHV-4, a low viral load in nasal secretions may be consistent with a lack of viral replication, representing non-replicating, non-infectious virus. Such animals, even if EHV-1 was not detected by either a nasopharyngeal or nasal swab, would generally not represent a risk of infection to other horses.

To minimize temporal variation in disease stages, only horses presented for acute onset of either fever or neurological signs were selected as true EHV-1 index cases in this study. Furthermore, the selection of subclinical cases was based on the absence of documented clinical signs for the duration of 14 days around the time of sample collection. The study showed differences in viral loads determined by real-time PCR between disease stages in adult horses as well as between clinically and subclinically infected horses. All horses presented for fever had detectable EHV-1 in both blood samples and nasopharyngeal swabs, but viral loads were significantly higher in blood. Our results are in agreement with experimental studies showing that viremia commonly coincides with the second pyrexia peak in EHV-1 infection. Peak shedding in nasal secretions is often documented during the first pyrexia peak, whereas the virus is often not detectable by PCR molecular detection methods in blood collected at the same time. Viral loads in blood and nasal secretions of febrile horses seem to be different from the viral loads encountered in neurological and subclinical horses.

Neurological deficits associated with EHV-1 infection are reported to be during the viremic phase of infection; the interval between infection and subsequent onset of neurological disease is usually between 6 and 10 days. The 15 neurological horses in our study showed low viral loads in blood and up to 1,000,000-fold higher viral loads in nasal secretions. In addition, viral loads in the nasal secretions of neurological horses were significantly higher than those of either febrile or subclinically infected horses. These findings are in contrast with the previously held but erroneous assumption that neurological horses infected with EHV-1 are no longer shedding virus. Recent outbreaks at racetracks, riding schools, and veterinary hospitals prove the contagiousness of neurologically affected horses.

The prevalence of EHV-1 in the Thoroughbred population from which the 41 subclinically infected horses used in this study originated approached 20% (data not shown). The results showed that the majority of subclinical adult horses were shedding only low viral loads in nasal secretions, and the viral kinetics were distinct from those seen in clinically affected horses. Documentation of the viral state was not an objective of this study; however, based on the low viral loads present in nasal secretions of subclinically infected horses, we assume that the detected virus represents either low-level, transient carriage of virus from reactivation/exposure or non-replicating, latent virus. The results of this study strongly suggest that the random testing of healthy horses for EHV-1 by PCR should be avoided, because practicing veterinarians and regulatory officials who receive positive PCR test results on samples they submit may be unaware of the complexities involved in test interpretation. This may lead them to make inappropriate decisions regarding quarantine of equine facilities or cancellation of competitions. The situation is likely different when healthy horses determined to be at high risk of exposure are tested for surveillance purposes during active outbreaks of clinical EHV-1 infection. Under such circumstances, horses that test positive by PCR on nasal secretions should be isolated and closely monitored for the development of clinical signs, because the viral load pattern of infected horses during the early
incubation period is similar to that of subclinical carriers. Follow-up assessment of viral loads in blood and nasal secretions can be used to help guide modification of infectious disease-control measures, including lifting of quarantine, for individual horses that test negative on a subsequent sample.

References and Footnotes


aFox Converting Inc., Green Bay, WI 54301.
bCorbett Life Science, Sydney, Australia NSW 2137.
cQIAGEN Inc., Valencia, CA 91355.
dApplied Biosystems, Foster City, CA 94404. Vol. 53, No. 1