Diagnostic sensitivity of nasopharyngeal and nasal swabs for the molecular detection of EHV-1

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EQUINE herpesvirus type 1 (EHV-1) is a major equine pathogen, responsible for well documented syndromes of respiratory disease, abortion, neonatal foal death and myeloencephalopathy (Van Maanen 2002). Recent outbreaks at riding schools, racetracks and veterinary hospitals throughout North America and Europe have highlighted the importance of early detection of EHV-1. The traditional approach of virus isolation has been supplanted in recent years by more sensitive and rapid molecular assays. Real-time PCR has become the most reliable detection method for the routine diagnosis of EHV-1 (Allen and Breathnach 2006, Hussey and others 2006, Pusterla and others 2006). Rapid molecular diagnosis is crucial to allow the prompt implementation of measures to prevent exposure of other horses and thereby minimise outbreaks. Samples of blood are routinely collected in the clinical setting to document viraemia, while secretions from the upper respiratory tract are taken to investigate the shedding of EHV-1. While the collection of whole blood rarely poses a problem, less compliant horses frequently resist the 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 using short nasal swabs. However, the diagnostic sensitivity of nasal swabs in the molecular detection of EHV-1 has, to the authors' knowledge, not been reported. The objective of this study was to compare the diagnostic sensitivity of nasal swabs with the traditionally recommended nasopharyngeal swabs for the molecular detection of EHV-1.

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 programme, a number of EHV-1 index cases and potentially exposed horses were surveyed over a period of three weeks following the diagnosis of EHV-1 myeloencephalopathy in a three-year-old thoroughbred gelding. Dual samples (nasopharyngeal and nasal swabs) were collected from 119 adult thoroughbred horses and 27 adult horses belonging to other breeds (quarter horse, American paint horse, Appaloosa). None of the 146 horses was febrile or displayed neurological signs at the time of sample collection. Nasopharyngeal swabs with rayon tips (40 cm swab; Fox Converting Swabs) were advanced rapidly through the ventral meatus of the right nasal passage to the pharynx and gently rotated for 10 seconds before being withdrawn. The same type of swab with the handle cut down to 10 cm in length was used to swab the ventral aspect of the left nasal passage. The nasal swab was advanced 10 cm into the ventral meatus and gently rotated for 10 seconds before being withdrawn. Thereafter, the swabs were placed in sterile prelabelled 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 hours of collection.

Five millilitres of phosphate-buffered saline solution (PBS) was added to the conical tube containing each swab. Each swab was vortexed for 10 seconds, inverted and centrifuged at 16,000 *g* for five minutes in order to retrieve a cell pellet. After removing the swab and supernatant, each pellet was resuspended in 400 µl of PBS before nucleic acid extraction. In order to minimise contamination, all pipetting steps were performed under laminar flow. Nucleic acid extraction was performed using an automated nucleic acid extraction system (CAS-1820 X-tractor Gene; Corbett Life Science) according to the manufacturer's recommendations.

To determine the efficiency of nucleic acid extraction from the two sample types, all the samples were analysed for the presence of the equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH) gene as described by Pusterla and others (2006). This target gene is routinely used in the authors' laboratory as a housekeeping gene for quality control of samples. Concurrently, all the samples were assayed for the presence of the glycoprotein B (gB) gene of EHV-1 using the real-time TaqMan PCR assay described by Pusterla and others (2006). The samples were amplified in a combined thermocycler/fluorometer (7900 HTA; Applied Biosystems) with the standard thermal cycling protocol: two minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The results were expressed as cycle threshold (C_t) values, which represent the fractional cycle number at which the fluorescence passes the fixed threshold, that is, when 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 eGAPDH and expressed as EHV-1 gene copies per million epithelial cells (Pusterla and others 2005). Any statistical difference ($P<0·05$) in the extraction efficiency of the eGAPDH gene and EHV-1 gB gene between the two sample types was determined using the Mann-Whitney U test.

DNA was successfully extracted from all 146 nasopharyngeal and nasal swabs. The mean (sd) C_t value for eGAPDH in the nasopharyngeal swabs and in the nasal swabs was 21·93 (2·08) and 22·81 (2·43), respectively. The difference in C_t values for eGAPDH 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 positive for EHV-1 in both samples (Table 1), with a mean C_t value for the gB gene of 33·66 (5·81) in the nasopharyngeal swabs and 32·87 (4·78) in the nasal swabs. The difference in C_t 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 $1·9 \times 10^8$ (median 9439) copies per million cells for the nasopharyngeal swabs and from 131 to $1·9 \times 10^6$ (median 6464) copies per million cells for the nasal swabs, respectively ($P>0·05$). Five horses tested EHV-1 positive only on nasopharyngeal swabs (C_t value 37·45 [2·46]), while nine horses tested EHV-1 positive only on nasal swabs (C_t value 35·81 [4·29]) ($P>0·05$). The median absolute viral load between the two sample types was similar, with 2555 and 2400 copies per million cells for nasopharyngeal and nasal swabs, respectively. When the combined (nasopharyngeal and nasal) C_t values of the gB gene for the 19 dual positive samples were compared with the C_t values of the 14 single positive nasopharyngeal and nasal samples, the difference was statistically significant ($P=0·01$). A significant difference ($P=0·006$) was also determined when absolute EHV-1 load was compared between the

TABLE 1: Results of real-time PCR for equine herpesvirus type 1 (EHV-1) in nasopharyngeal and nasal swabs collected from 146 adult horses

	Nasopharyngeal swab	
	EHV-1 PCR positive	EHV-1 PCR negative
Nasal swab		
EHV-1 PCR positive	19	9
EHV-1 PCR negative	5	113

Veterinary Record (2008) **162,** 520-521

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19 dual positive samples (median load 7952 EHV-1 copies per million cells) and the 14 single samples (median load 511 EHV-1 copies per million cells).

It is extremely important that nasal secretions are collected from an appropriate anatomical site and at the proper time after infection, because EHV-1 is shed for only a short period of time (Van Maanen 2002). Although the collection of a nasal swab for the molecular detection of EHV-1 is routinely performed by equine practitioners, due to better tolerance by the horse 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 C_t values for the eGAPDH, which is a prerequisite for sensitive and accurate detection of the target gene (the EHV-1 gB gene).

For the molecular and conventional detection of viral and bacterial pathogens, swab material should be made out of sterile cotton, rayon, polyester or dacron. Calcium alginate swabs and wooden-shafted swabs should be avoided because they are toxic for many bacteria and enveloped viruses (Hanson and Schipper 1976, Crane and others 1980, Lauer and Masters 1988, Wadowsky and others 1994, Cloud and others 2002). The tip of the swabs used in the present study was made out of rayon, a material shown not to negatively influence PCR results. When swabs are used to collect secretions and exfoliating epithelial cells from the upper airways of horses, a minimum contact time of 10 seconds is recommended to allow maximum absorption of secretions. Further, rotating the swab against the respiratory mucosa will increase the retrieval of epithelial cells. The collection of both mucosal secretions and epithelial cells by either nasopharyngeal or nasal swabbing is highly desirable, since viral respiratory pathogens are found either free in the mucus or within epithelial cells. Although both nasopharyngeal and nasal swabs yielded similar nucleic acid recovery in this study, the authors speculate that nasal swabs are more likely to contain target DNA, since mucus and exfoliated cells from the upper airways are drained via the ventral meatus by gravity and mucociliary clearance.

Viral state and load and disease state are likely to influence the molecular detection of EHV-1. In 19 of the horses sampled, EHV-1 was detected concurrently from nasopharyngeal and nasal swabs, with no statistical difference in C_t values. An additional 14 horses tested PCR positive in either of the sample types, with almost twice as many nasal swabs (nine horses) testing PCR positive alone than nasopharyngeal swabs (five horses). The C_t 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 appear to be diagnostically more sensitive in the detection of a 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 suspended in 80 µl of sterile water) or to the small amount of DNA (1 µl) used for each PCR. The use of triplicates for each sample and/or larger volumes 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 equine herpesvirus type 4, a low viral load in nasal secretions may be consistent with a lack of viral replication, representing non-replicating, non-infectious virus (Pusterla and others 2005). Such animals, even if not detected by either a nasopharyngeal or a nasal swab, would generally not represent a risk of infection to other horses.

In conclusion, the present study shows that nasal swabbing represents a viable alternative to the less well tolerated nasopharyngeal swabbing technique for the molecular detection of EHV-1 in horses. The use of nasal swabs from index and exposed horses will increase the incidence of EHV-1 in the tested population. The data generated in this study should not be extrapolated to other equine viral or bacterial pathogens associated with infection of the upper respiratory tract.

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