Use of viral loads in blood and nasopharyngeal secretions for the diagnosis of EHV-1 infection in field cases

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THE increasing application of sensitive molecular diagnostic techniques for the 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 PCR assays targeting various genes of equine herpesvirus type 1 (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 (Sharma and others 1992, Borchers and Slater 1993). In recent years, novel molecular platforms, such as real-time PCR, have enabled the study of the viral kinetics of EHV-1 in respiratory secretions and blood samples after experimental infection (Allen and Breathnach 2006, Hussey and others 2006). However, the determination and reporting of EHV-1 viral loads in samples submitted by veterinarians from field cases is not current practice in the molecular diagnostic setting to enable practitioners to determine disease stage or response to antiviral therapy. The objective of this study was to determine EHV-1 viral loads by real-time PCR in blood and nasopharyngeal secretions collected from clinically affected and subclinically infected adult horses.

The study population comprised 27 adult horses with clinical signs consistent with EHV-1 infection. These horses were presented either to primary care veterinarians or the Veterinary Medical Teaching Hospital, University of California at Davis, because of acute onset of fever (fever group, 12 horses) or neurological signs (neurological group, 15 horses). The 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 comprised 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. The selection of these study horses was based on the molecular detection of EHV-1 in blood samples or nasopharyngeal secretions and the absence of fever (<38·5°C), nasal discharge, cough and neurological signs. Only horses with a documented absence of clinical signs seven days before and seven days after sample collection were included in the study. This precluded the inclusion of horses with clinical disease.

Whole anticoagulated blood samples (BD Vacutainer) and nasopharyngeal swabs (Fox Converting Swabs) were available from each horse for molecular testing. DNA from blood samples and nasopharyngeal swabs was extracted using a commercial DNA kit (DNeasy Tissue Kit; QIAGEN) according to the manufacturer's instructions. All samples were assayed for the presence of the equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH) gene and the glycoprotein B (gB) gene of EHV-1 using previously described real-time TaqMan PCR assays (Pusterla and others 2006). 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 cells (peripheral blood or nasopharyngeal epithelial cells) as reported previously by Pusterla and others (2005). Statistical differences ($P<0·05$) in EHV-1 viral loads between the groups were determined using the Mann-Whitney U test.

EHV-1 was detected in samples of both blood and nasopharyngeal secretions from all the febrile horses (Table 1). Viral loads were significantly higher ($P=0·003$) in blood samples (mean [sd] $1·7 \times 10^4$ [$9·7 \times 10^3$] gene copies/million cells) than in nasopharyngeal secretions ($2·8 \times 10^3$ [$2·5 \times 10^3$] gene copies/million cells). Only nine of the 15 neurological horses tested PCR positive in blood samples, whereas EHV-1 was detected in the nasopharyngeal secretions of all 15. When comparing only the samples from the nine neurological horses with dual positive results, viral loads in nasopharyngeal secretions ($1·4 \times 10^6$ [$1·2 \times 10^6$] 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, while 40 of the 41 subclinical horses tested PCR positive in nasopharyngeal 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. Significant differences were also found for viral loads in nasopharyngeal secretions between neurological horses and febrile horses ($P=0·001$) and between neurological and subclinically infected horses ($P=0·001$). No significant difference was found in the nasopharyngeal viral loads between febrile and subclinical horses ($P>0·05$).

It is routinely assumed that the molecular detection of EHV-1 in blood samples and nasopharyngeal secretions indicates viraemia from active infection and the shedding of infectious virus, respectively. It must be kept in mind that PCR assays used for molecular diagnosis do not allow any interpretation of the biological state of the virus (that is, lytic, non-replicating or latent). Recent advances in molecular technology allow discrimination between EHV-1 viral states by (i) targeting several genes (for example, glycoprotein, latency-associated transcripts); (ii) detecting viral genomic DNA and transcriptional activity of the target genes at the messenger RNA level; and (iii) using absolute quantification. Standardised use of these novel PCR assays by both commercial and research laboratories is necessary for more accurate investigation of disease outbreaks in the future. For example,

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TABLE 1: Quantitative PCR results for EHV-1 in blood and nasopharyngeal secretions collected from neurological, febrile and subclinical horses

Group (n)	Sample type	PCR positive results (%)	Viral loads (gene copies/million cells) Minimum	Maximum	Median	Mean
Febrile (12)	Blood	12 (100)	$2·3 \times 10^3$	$2·6 \times 10^4$	$2·0 \times 10^4$	$1·7 \times 10^4$
	Nasopharyngeal secretions	12 (100)	$2·5 \times 10^2$	$6·7 \times 10^3$	$2·1 \times 10^3$	$2·8 \times 10^3$
Neurological (15)	Blood	9 (60)	0	$2·5 \times 10^2$	$1·4 \times 10^2$	$1·6 \times 10^2$
	Nasopharyngeal secretions	15 (100)	$1·3 \times 10^4$	$3·6 \times 10^6$	$5·1 \times 10^5$	$1·0 \times 10^6$
Subclinical (41)	Blood	5 (12)	0	$4·9 \times 10^2$	0	$4·5 \times 10^1$
	Nasopharyngeal secretions	40 (97)	0	$9·8 \times 10^5$	$3·6 \times 10^3$	$8·6 \times 10^4$

viral load testing is used routinely in selected human infectious diseases, such as HIV and hepatitis C to determine prognosis and disease stage, and to monitor response to therapy (Mellors and others 1995, 1996, Saldanha and others 1999). The concept of viral loads has been used diagnostically in EHV-4-infected horses in order to discriminate between lytic and non-lytic infection (Pusterla and others 2005).

In order to minimise 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. Further, the selection of subclinical cases was based on the absence of documented clinical signs for 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 clinical and subclinically infected horses. All horses presented for fever had detectable EHV-1 in both blood samples and nasopharyngeal swabs, with significantly higher viral loads in blood. These results are in agreement with experimental studies showing that viraemia commonly coincides with the second peak of pyrexia in EHV-1 infection (Hussey and others 2006). Peak shedding in nasopharyngeal secretions is often documented during the first pyrexia peak, while virus is often not detectable by PCR molecular methods in blood collected at the same time. Viral loads in blood and nasopharyngeal secretions of febrile horses appear to be different from the viral loads encountered in neurological and subclinical horses.

Neurological deficits associated with EHV-1 infection appear during the viraemic phase of infection and the interval between infection and subsequent onset of neurological disease is usually between six and 10 days (Van Maanen 2002). The 15 neurological horses showed low viral loads in blood and up to one million-fold higher viral loads in nasopharyngeal secretions. These findings are in contrast with the previously held erroneous assumption that neurological cases infected with EHV-1 are no longer shedding virus. Recent outbreaks at racetracks, riding schools and veterinary hospitals provide ample documentation of the contagiousness of neurologically affected horses (Kohn and others 2006, Henninger and others 2007). The silent circulation of EHV-1 in horse populations has been well documented in the case of mares and foals (Gilkerson and others 1999). Furthermore, the prevalence of nasal viral detection determined by PCR has been shown to approach 4 per cent in healthy weanling and juvenile horses at major equine shows and sales (Yactor and others 2006).

The prevalence of EHV-1 in the thoroughbred population from which the 41 subclinically infected horses used in this study originated approached 20 per cent (data not shown). The results showed that the majority of subclinical adult horses were shedding only low viral loads in nasopharyngeal 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 the nasopharyngeal secretions of subclinically infected horses, it can be assumed that the detected virus represents either low-level, transient carriage of virus from reactivation, or exposure, or non-replicating, latent virus.

The results of this study strongly suggest that the random testing of normal horses for EHV-1 by PCR should be avoided, since practising veterinarians and regulatory officials who receive positive PCR test results on samples they submit may be unaware of the complexities involved in test interpretation, leading them to make inappropriate decisions regarding quarantine of equine facilities or cancellation of competitions. The situation is likely to be 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 nasopharyngeal secretions should be isolated and monitored closely for the development of clinical signs, since 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 nasopharyngeal secretions can be used to help guide the modification of infectious disease control measures, including lifting of quarantine, for individual horses that test negative on a subsequent sample.

In conclusion, the present study shows significant differences in EHV-1 viral load signatures in blood and nasopharyngeal secretions between clinically affected and subclinically infected horses, and between those in the febrile and neurological stages of the disease. These results support the need for a consensus on the use and interpretation of molecular diagnostic techniques in the evaluation of field cases of suspected EHV-1 infection. Diagnostic laboratories should consider reporting quantitative information regarding EHV-1 viral loads in blood and nasopharyngeal secretions 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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