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Detection of equine herpesvirus in horses with idiopathic keratoconjunctivitis and comparison of three sampling techniques

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Abstract

Objectives To determine the role of equine herpesvirus (EHV) in idiopathic keratoconjunctivitis in horses and to determine whether sample collection method affects detection of EHV DNA by quantitative polymerase chain reaction (qPCR).

Animals studied Twelve horses with idiopathic keratoconjunctivitis and six horses without signs of ophthalmic disease.

Procedures Conjunctival swabs, corneal scrapings, and conjunctival biopsies were collected from 18 horses: 12 clinical cases with idiopathic keratoconjunctivitis and six euthanized controls. In horses with both eyes involved, the samples were taken from the eye judged to be more severely affected. Samples were tested with qPCR for EHV-1, EHV-2, EHV-4, and EHV-5 DNA. Quantity of EHV DNA and viral replicative activity were compared between the two populations and among the different sampling techniques; relative sensitivities of the sampling techniques were determined.

Results Prevalence of EHV DNA as assessed by qPCR did not differ significantly between control horses and those with idiopathic keratoconjunctivitis. Sampling by conjunctival swab was more likely to yield viral DNA as assessed by qPCR than was conjunctival biopsy. EHV-1 and EHV-4 DNA were not detected in either normal or IKC-affected horses; EHV-2 DNA was detected in two of 12 affected horses but not in normal horses. EHV-5 DNA was commonly found in ophthalmically normal horses and horses with idiopathic keratoconjunctivitis.

Conclusions Because EHV-5 DNA was commonly found in control horses and in horses with idiopathic keratoconjunctivitis, qPCR was not useful for the etiological diagnosis of equine keratoconjunctivitis. Conjunctival swabs were significantly better at obtaining viral DNA samples than conjunctival biopsy in horses in which EHV-5 DNA was found.

Key Words: conjunctival biopsy, conjunctival swab, corneal scraping, EHV, infectious keratitis, qPCR

INTRODUCTION

Herpesviruses are an important cause of ocular disease in many species. Both herpes simplex virus type 1, an alphaherpesvirus, and Epstein–Barr virus (EBV), a gammaherpesvirus, have been linked to recurrent keratoconjunctivitis in humans,[1,2] and alphaherpesviruses are important causes of keratoconjunctivitis in cats, dogs, and cattle.[3–5] To the authors' knowledge, the role of the alphaherpesvirus EHV-1 in equine keratoconjunctivitis has not been specifically investigated; however, the association between the gammaherpesvirus EHV-2 and keratoconjunctivitis in horses has been studied.[6] Young horses experimentally infected with EHV-2 developed keratoconjunctivitis and EHV-2 was detected in the ocular secretions of foals and horses with spontaneous keratoconjunctivitis.[7–10] Clinical response in foals with keratoconjunctivitis and treated with antiviral medications

also suggests a viral etiology.[8] However, authors of a recent study concluded that EHV-2 was less frequently detected with nested PCR in the ocular secretions of horses with keratoconjunctivitis than in normal horses.[11]

Because herpesviruses are obligate intracellular infections agents, quantification of EHV DNA using quantitative PCR (qPCR) may be more sensitive and reliable when performed on cellular samples such as corneal scrapings or conjunctival biopsies rather than ocular secretions. However, sampling technique may be influenced by the phase of infection. During latent infection cycles, viral DNA is intracellular. Conversely, during lytic infection cycles, viral DNA could be in secretions or intracellularly. Although research assessing the association of EHV-2 with idiopathic keratoconjunctivitis supports an etiological role, the ubiquity of equine herpesvirus infections and possibly the choice of ocular sample used have led to some confusion in interpretation of results. Because equine herpesviruses are endemic in equine populations,[12,13] PCR detection of EHV DNA in an ocular sample of a horse affected with keratoconjunctivitis is not necessarily indicative of a causal relationship. However, use of qPCR makes it possible to compare viral load and replicative activity between affected and control horses. For equine alphaherpesviruses, higher DNA viral loads are indicative of active lytic infection, whereas lower viral loads are more supportive of latent or abortive infection.[14,15] We hypothesized that finding high EHV loads in ocular samples of affected horses may also suggest active infection in the eyes of such horses.

Selection of ocular sample type affects the success of herpesvirus detection.[16] The study which concluded that EHV-2 infection was less common in the eyes of horses with keratoconjunctivitis utilized ocular swabs;[11] however, corneal scrapings or conjunctival biopsies may be more appropriate samples for the consistent detection of herpesviruses because herpesviruses directly infect corneal cells *in vitro*,[17] and PCR testing of corneal scrapings is a successful diagnostic test in clinical cases of herpetic keratitis.[18] The close association of many herpesviruses with lymphoid and/or epithelial tissues makes conjunctiva another attractive site for diagnostic sampling.[19] While ocular swabs are certainly used as diagnostic samples, we hypothesized that dilution of the sample by increased ocular secretions with eye irritation as well as potential PCR inhibitors in the tear film may contribute to decreased sensitivity when these samples are used. The sensitivity of swabs may also be affected by inhibitors in the tear film. In our study, the samples were not washed to minimize this effect.

The purpose of this study was to determine whether detection of high viral loads and replicative activity of EHV was associated with idiopathic keratoconjunctivitis in horses and to determine whether ocular sampling method affects detection of EHV by qPCR.

MATERIALS AND METHODS

Animals

Twelve client-owned horses diagnosed with idiopathic keratoconjunctivitis by a board-certified veterinary ophthalmologist at the University of California Davis William Pritchard Veterinary Medical Teaching Hospital (UCD VMTH) were enrolled in the study after obtaining informed owner consent. All of the horses were presented for eye disease only and were otherwise without any signs of systemic illness. They all received a complete general physical examination by an equine internal medicine faculty member or resident. Detailed histories, descriptions of the clinical lesions, and digital photographs were collected for both eyes of each horse. After routine sedation, a conjunctival swab, corneal scraping, and conjunctival biopsy sample were collected for quantification of viral DNA from EHV-1, EHV-2, EHV-4, and EHV-5 by use of qPCR. The conjunctival swab sample was collected from the conjunctival fornix of the lower eyelid using a sterile, rayon tipped applicator (Puritan Medical Products Co. LLC, Guilford, ME, USA). Proparacaine, 0.5%, (Akorn, Inc., Lake Forest, IL, USA) was then applied to the ocular surface. The corneal scraping was taken from an area judged by the examining ophthalmologist to be the most severely affected. Finally, a small 'snip' biopsy sample of conjunctiva was obtained from an area near the fornix of the lower eyelid. All samples were placed in a red top tube and immediately delivered to the laboratory for qPCR processing. As part of the typical diagnostic approach for horses with keratoconjunctivitis, samples were then collected from the corneal lesions and submitted for aerobic bacterial culture, fungal culture, and cytologic assessment. In bilaterally affected horses, the eye judged to be more severely affected was sampled. If no known cause of keratoconjunctivitis was identified, the horse was diagnosed with idiopathic keratoconjunctivitis. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California.

Six client-owned horses that were being euthanized for reasons unrelated to this study were examined by a board-certified veterinary ophthalmologist and determined to be free of evidence of ocular disease. Immediately after euthanasia, these horses had conjunctival swabs, conjunctival biopsies, and corneal scrapings taken from one eye and submitted to the same testing with regard to qPCR, cytology, and culture and sensitivity as performed for horses with idiopathic keratoconjunctivitis.

qPCR analysis

Nucleic acid extraction from conjunctival swabs, corneal scrapings, and conjunctival biopsies was performed using the recently established and validated automated nucleic acid extraction CAS-1820 X-tractor Gene (Corbett Life Science, Sydney, NSW, Australia). The DNA quality was

verified by quantitating the housekeeping equine gene glyceraldehyde-3-phosphate dehydrogenase (eGAPDH).

Real-time TaqMan PCR assays (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) targeting the glycoprotein B gene of EHV-1, EHV-2, EHV-4, and EHV-5 were used to analyze all samples as previously reported.[15] Quantification of the results was performed using pre-existing standard curves for EHV-1, EHV-2, EHV-4, EHV-5, and eGAPDH. Results were reported as number of EHV-1, EHV-2, EHV-4, and EHV-5 target genes per million equine cells.

Statistical analysis

Exact Friedman one-way analysis of variance was used to evaluate differences in viral load between the three sampling methods; exact Wilcoxon signed-rank tests for paired data were used for post hoc comparisons. Exact Wilcoxon–Mann–Whitney tests were used to evaluate differences in the distributions of viral load between the two study groups (idiopathic keratoconjunctivitis and unaffected) under individual sampling methods. Statistical significance was defined as $P < 0.05$ for all analyses.

RESULTS

Age of the 12 horses in the affected group ranged from 4 to 27 years with a median of 16 years. Eight breeds were represented including Arabian ($n = 3$), Quarter Horse ($n = 3$), Thoroughbred ($n = 1$), Morgan ($n = 1$), Percheron ($n = 1$), Shire ($n = 1$), Haflinger ($n = 1$), and Quarter Horse/Standardbred cross ($n = 1$). There were six geldings and six mares in the affected group. The control group ranged in age from 1 to 27 years with a median of 14.5 years. There were four breeds including Arabian ($n = 3$), Thoroughbred ($n = 2$), and Quarter Horse ($n = 1$). Four of the control horses were mares, one was a stallion, and one was a gelding. Typical lesions of affected horses consisted of superficial corneal vascularization with or without areas of punctate superficial corneal ulceration (Figs 1 and 2). Of the 12 horses with idiopathic keratoconjunctivitis, four were affected bilaterally, four had lesions of the right eye only, and four had lesions of the left eye only. Fungal and aerobic bacterial cultures failed to reveal organisms in samples collected from any horse in either group. Likewise, cytologic assessment failed to diagnose a cause for keratoconjunctivitis in affected horses or any abnormalities in horses from the control group. Viral DNA from EHV-1 or EHV-4 was not detected in any horse from the control group or those with idiopathic keratoconjunctivitis. Results of qPCR by all three sampling techniques for affected and control horses are shown in Tables 1 and 2, respectively. Considering all samples collected from the 12 affected horses, no EHV DNA of any type was detected in four horses, and EHV-5 was detected in four horses, from two of which EHV-2 DNA was also detected by at least one sampling method. In all samples collected from the six control horses, EHV DNA was not detected in three horses while EHV-5 was detected in the remaining three horses. There was no significant difference in EHV-5 detection rates between affected horses and controls ($P = 0.76$).

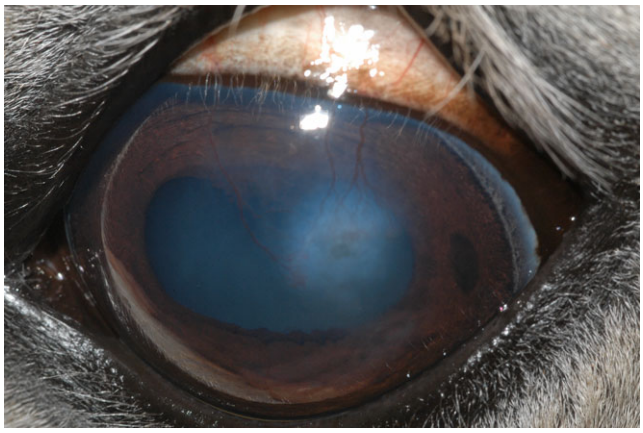

Figure 1. Affected horse number 1 OS. This horse exhibited faint superficial corneal fibrosis and superficial corneal vascularization OU. Neither cornea retained fluorescein stain. Quantitative PCR failed to detect EHV DNA of any strain in any of the three sample methods tested.

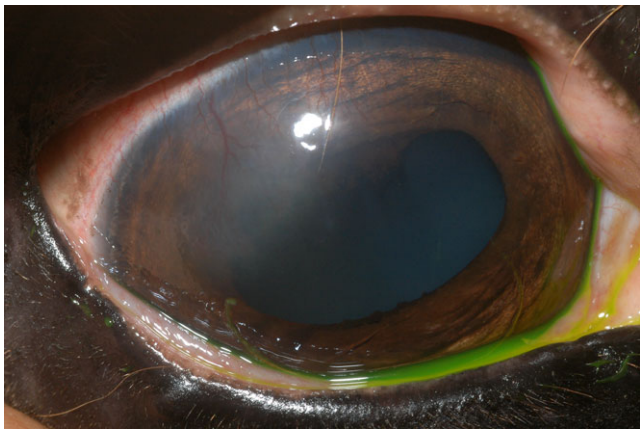

Figure 2. Affected horse number 10 OD. Clinical signs observed OU in this horse were very similar to those seen in the horse in Figure 1. Neither cornea retained fluorescein stain. However, unlike affected Horse # 1, EHV-2 and EHV-5 DNA were detected by all three sampling methods using qPCR.

Considering the two horses with keratoconjunctivitis and in which EHV-2 DNA was detected, EHV-2 qPCR results for all three sample types were concordant in one horse; only the swab failed to reveal EHV-2 DNA in the other horse. Of these two horses, one horse (affected #10) was strongly positive for EHV-2 by conjunctival swab (2,527,147 viral DNA copies per million cells) and corneal scraping (882,012,758 viral DNA copies per million cells), but relatively weakly positive on conjunctival biopsy (12,930 viral DNA copies per million cells). The other

Table 1. Quantitative polymerase chain reaction (qPCR) results for 12 horses with idiopathic keratoconjunctivitis

Affected horse no.	Age in years	Horse breed	Horse gender	Sample method	EHV-2 Viral DNA copies per million cells, log 10	EHV-5 Viral DNA copies per million cells, log 10
1	11	Arabian	Gelding	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
2	4	QH	Female	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
3	8	Haflinger	Gelding	Swab	–	6.01
				Scrape	–	7.74
				Biopsy	–	6.01
4	17	Shire	Female	Swab	–	4.63
				Scrape	4.42	4.15
				Biopsy	2.07	2.72
5	16	TB	Gelding	Swab	–	4.58
				Scrape	–	5.16
				Biopsy	–	–
6	12	Percheron	Female	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
7	20	Morgan	Female	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
8	16	QH	Gelding	Swab	–	4.58
				Scrape	–	5.79
				Biopsy	–	2.21
9	19	QH/Std	Gelding	Swab	–	–
				Scrape	–	–
				Biopsy	–	5.49
10	17	QH	Gelding	Swab	6.40	5.97
				Scrape	8.95	5.80
				Biopsy	4.11	4.11
11	13	Arabian	Female	Swab	–	7.45
				Scrape	–	6.06
				Biopsy	–	–
12	27	Arabian	Female	Swab	–	–
				Scrape	–	4.47
				Biopsy	–	–

Negative results are listed as –.

horse (affected #4) was negative on conjunctival swab and only weakly positive on corneal scraping (26,405 Viral DNA copies per million cells) and conjunctival biopsy (118 Viral DNA copies per million cells). Considering the eight horses with keratoconjunctivitis and in which EHV-5 DNA was detected, EHV-5 qPCR results for all three sample types were concordant in four horses, concordant in two sample types in two horses (the corneal scrape and conjunctival biopsy in one and the conjunctival swab and corneal scrape in the other), and positive in only one sample type for two horses (one each for the conjunctival biopsy and corneal scrape). Results of EHV qPCR were concordant in all sample types collected from all control horses. Considering horses from which EHV-5 was detected, no significant difference among sampling methods was detected for horses with ($P = 0.14$) or without ($P = 0.19$) keratoconjunctivitis. The sample size was too small to statistically compare the effect of sampling method on the two horses with keratoconjunctivitis and from which EHV-2 was detected. Considering control and affected horses together, results did vary among the three sampling methods ($P = 0.035$), with conjunctival swabs more likely than conjunctival biopsies to yield viral DNA ($P = 0.027$).

DISCUSSION

Keratitis is a common problem in horses. Numerous causes have been identified including bacterial infection, fungal infection, viral infection, and autoimmune disease.[20,21] While diagnosis is often straightforward, many cases remain idiopathic. These keratopathies are often painful and chronic, and failure to identify a cause is frustrating to clients and clinicians. Herpesviruses has been demonstrated or suspected as the cause of keratoconjunctivitis in a wide number of species including horses.[3–10]

Table 2. Quantitative polymerase chain reaction (qPCR) results for six horses free of ocular disease

Control horse no.	Age in years	Horse breed	Horse gender	Sample method	EHV-2 Viral DNA copies per million cells, log 10	EHV-5 Viral DNA copies per million cells, log 10
1	11	TB	Female	Swab	–	4.07
				Scrape	–	–
				Biopsy	–	3.13
2	1	QH	Female	Swab	–	5.97
				Scrape	–	5.80
				Biopsy	–	4.11
3	23	TB	Female	Swab	–	7.80
				Scrape	–	7.04
				Biopsy	–	5.06
4	27	Arabian	Female	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
5	12	Arabian	Male	Swab	–	–
				Scrape	–	–
				Biopsy	–	–
6	17	Arabian	Gelding	Swab	–	–
				Scrape	–	–
				Biopsy	–	–

Negative results are listed as –.

Furthermore, numerous studies from the United States or Europe have used a variety of diagnostic testing procedures to demonstrate presence of EHV-2 in horses with idiopathic keratoconjunctivitis.[8–11] Although presenting clinical signs of the affected horses in the present study were similar to those reported in other studies, we did not find qPCR detection of EHV-2 DNA to be associated with idiopathic equine keratoconjunctivitis. Our findings are consistent with one study where only four of 48 horses displaying surface ophthalmic disease tested positive for EHV-2 using nested PCR.[11] The discrepancy between our findings and other previous studies is most likely due to the fact that detection of EHV-2 by use of PCR or nested PCR,[9,11] fluorescent antibody,[10] or viral culture[8] in horses with keratoconjunctivitis does not prove causality, as EHV-2 is widespread in horses without clinical signs.[11,22–25] Quantitative PCR allows for assessment of viral load and replicative activity both of which are directly associated with lytic cycles, although this in itself does not prove causality.

By contrast, we detected EHV-5 DNA in eight of twelve affected horses; however, it was also found in three of six control horses making any determination of causality impossible. Finally, on the basis of presenting clinical signs, it was not possible to differentiate horses in which EHV-2 or EHV-5 DNA was ultimately detected from those in which EHV DNA was not detected (see Figs 1 and 2). As with many clinical trials, one weakness of our study was the relatively small sample size. Admittedly, if more horses had been included, the results may have been different.

In our study, there was no significant difference between sample collections methods when affected horses or control horses were considered within their respective groups. However, considering all horses irrespective of disease status, conjunctival swabs were more likely than conjunctival biopsies to yield viral DNA when subsequently tested using qPCR. Our findings in this regard were consistent with findings from a study where detection rates of EHV-1 were similar for nasal swabs and the more invasive nasopharyngeal swabs.[26] This may indicate that there is abundant herpetic DNA in the tear film or the readily exfoliated superficial conjunctival cell layers of horses. From a practical perspective, this is welcome as it suggests that the less invasive sampling technique can be relied upon to obtain sufficient viral DNA.

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