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Distribution and relevance of equine herpesvirus type 2 (EHV-2) infections

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Summary. Equine herpesvirus type 2 (EHV-2) is a slow-growing, cytopathogenic gammaherpesvirus, which is suggested to be ubiquitous in the equine population. However, its precise role as a pathogen and its tissue tropism remains uncertain. To estimate the prevalence of EHV-2 in Germany and to investigate the possible pathogenicity of the virus, peripheral blood leucocytes (PBL) from 172 horses were examined for EHV-2 DNA by a sensitive and specific nested PCR based on the *Eco*RI-N genomic fragment and by classical cocultivation. PBL samples from 51% of the horses were positive by PCR and virus was isolated from 31% of the horses by cocultivation. However, almost all animals were seropositive for EHV-2. This may indicate that PBL do not harbour EHV-2 indefinitely after infection. Furthermore, a correlation between clinical signs and EHV-2 as a causative agent could not be determined. Nevertheless, the prevalence of virus was high among horses with upper respiratory tract disease, abortion and severe ataxia.

The products of the second round of the PCR reactions showed size polymorphism. Sequencing of the products revealed that these size differences were due to repetition of the motif (AGACAGGGGCCATGCTGGC) between 9–16 times depending on the isolate, suggesting that the nested PCR might be a useful tool for the differentiation of EHV-2 isolates.

Introduction

Equine herpesvirus type 2 (EHV-2) is a slow-growing, cytopathogenic gammaherpesvirus [29], which is thought to be widespread in the equine population. EHV-2 has been isolated from 89% of 71 horses in the United States [15], 89% of 19 adult horses in England [23], and 77% of 13 horses in Switzerland [27]. Although EHV-2 has been implicated in upper respiratory tract disease, pyrexia, inappetance, lymphoadenopathy, immuno-suppression, general malaise and poor performance [3,17] (see also [6] for references), its

role as an equine pathogen remains uncertain. EHV-2 has been isolated from foals with keratoconjunctivitis superfacialis, leading to speculation that the virus may be a causative agent [8, 16, 32, 33]. However, EHV-2 has been isolated from tissues of apparently healthy horses [6, 14, 15, 23] and almost all adult horses surveyed were seropositive. Recently, it has been suggested that EHV-2 plays a role in transactivation and, possibly, reactivation of EHV-1 and EHV-4 [20, 36].

The site of EHV-2 latency is uncertain. However, like other gammaherpesviruses, EHV-2 has been isolated from circulating leucocytes [15, 23]. Whereas, viral antigen was detectable in pulmonary macrophages of horses with chronic pulmonary disease [27]. Dutta et al. [12] isolated EHV-2 from mammary gland macrophages by cocultivation. Other reports suggested that EHV-2 is B-cell tropic [1, 11]. Therefore, it has been suggested that in these subpopulations a latent EHV-2 infection can occur. However, results of Thein [30, 31] on experimentally infected horses with keratoconjunctivitis implicated that the ganglion ciliaris might be a site for latency. Although, EHV-2 has proved to be highly cell-associated, the virus could be isolated from nasal and ocular secretions [21]; (Borchers and Wolfinger, unpubl. results), where it is most probably in a cell-free form.

Considerable antigenic, genetic and biological heterogeneity among EHV-2 isolates have also been reported, and it has been suggested that variability among EHV-2 isolates may reflect variations in pathogenicity. Thus, differences were seen in tissue culture regarding rate of growth and plaque size [18, 19, 28, 34], in antigenic properties and in serological responses [19, 24]. Genomic heterogeneity was examined by Browning and Studdert [5] but could not be correlated with different biological properties.

The aim of the present study was two-fold: Firstly, to develop a specific and sensitive nested PCR for the detection of EHV-2 and to apply this technique to estimate the prevalence of EHV-2 in horses in the region of Berlin. Secondly, to further investigate the role of EHV-2 in pathogenicity.

Material and methods

Sample origin

Blood samples were collected from 172 horses in the Berlin area. This population includes 116 horses kept in 13 studs and 56 individual horses, not kept at stud. They were between 1 and 22 years of age and showed quite different clinical symptoms (Table 1): 76 animals (45%) of these had upper respiratory tract disease (group 1), 30 (18%) were members of a stud with abortion problems (group 2), 28 (15%) showed ataxia and chronical back problems (group 3), 19 (11%) had poor performance and general malaise (group 4), whereas 19 animals (11%) were clinically healthy (group 5).

DNA extraction from PBL

PBL were prepared from heparinised venous blood by centrifugation through a Ficoll gradient. For DNA extraction, 10^4–10^6 PBL were resuspended in 50–400 µl of proteinase

Table 1. Description of the test groups

Symptomatical groups		No. of horses	
		kept at stud	individual[a]
Group 1:	horses with respiratory tract diseases[b]	73	3
Group 2a:	mares with abortions between the sixth and ninth month of pregnancy	6	5
Group 2b:	horses of the studs the mares of 2a belong to	19	0
Group 3a:	horses with severe spinal ataxia	0	17
Group 3b:	horses with chronic inflammation of the muscles of the back	0	11
Group 4:	horses with fever, general malaise or poor performance	0	19
Group 5:	horses without clinical diseases	18	1
Total		116	56

[a]Individual horses were grouped according to their clinical signs
[b]Symptoms: Laryngitis follicularis, watery or mucoid nasal discharge, rare coughing

K digestion buffer (50 mM Tris pH 8.5; 1 mM EDTA; 0.5% Tween 20). After addition of 1 µl of proteinase K (10 mg/ml; Boehringer Mannheim) per 100 µl suspension, cells were incubated overnight at 56 °C and then heated at 60 °C for 10 min. The sample was finally boiled for 10 min at 95 °C to destroy the proteinase K, centrifuged for 1 min at 10 000 rpm and the supernatant was used for PCR.

Cocultivation

2.5×10^6 PBL were seeded with an equal number of equine dermal (ED) cells into tissue culture plates 5 cm in diameter (Nunc). The cells were observed microscopically daily for the appearance of viral cytopathic effect (CPE) and when no CPE was detectable after 7 days, the cultures were repassaged up to three times at weekly intervals with fresh ED cells.

Viral DNA preparation

EHV-2 isolates were propagated in ED cells. Virus from supernatants was pelleted by centrifugation through a 20% sucrose cushion (2 h at 27 000 rpm) and viral DNA was extracted with 1% sodium dodecyl sulfate (SDS) and proteinase K (Boehringer, Mannheim). Subsequently DNA was purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients.

Nested PCR

In order to detect EHV-2 DNA with high sensitivity and specificity we established a type-specific nested PCR based on a sequence upstream of the ORF coding for a product homologous to human Interleukin-10, which is located in the *Eco*RI-N-fragment of the EHV-2 strain [22]. The nested primer pairs were designed using a computer software package ('Oligo' version 4.0 for Macintosh). The outer primer pair was 20 nucleotides long

Table 2. Primer description

Primer (P)[a]	Sequence	Length[b]	mt[c](°C)
EHV-2 (P1)	5′-GGCACTGAAACCCCATACTG-3′	1 237 bp	64 °C
EHV-2 (P2)	5′-AAAACCATCCTGTCCAACCA-3′		64 °C
EHV-2 (P3)	5′-CCACTAACCCCCAACCTT-3′	621 bp	64 °C
EHV-2 (P4)	5′-CCTCTATCCTCACAACAG-3′		64 °C

[a]P1 is the upper primer of the outer pair, P2 is the lower primer of the outer pair, P3 is the upper primer of the inner pair, P4 is the lower primer of the inner pair

[b]Length of the amplificate

[c]*mt* Melting temperature

and the inner primer pair 18 nucleotides long. Primer sequences and positions are described in Table 2. PCR conditions were optimized using target DNA purified by equilibrium density centrifugation in caesium chloride-ethidium bromide gradients. DNA from EHV-2 (strains NHV, T16, T400) were used as positive controls and DNA from EHV-1 (strain Mar87), EHV-4 (strain T252) and EHV-5 (strain 2–141; kindly supplied by Dr. M.J. Studdert, University of Melbourne) served as negative controls.

The PCR mix (50 µl) contained 10 pg of purified DNA or 1 µg of cellular DNA, 0.4 µM of each primer, 0.2 mM dNTPs, 1.5 U Taq Polymerase (Perkin Elmer Cetus, Norwalk, USA) and 1x reaction buffer (Perkin Elmer Cetus, Norwalk, USA). The aqueous phase was covered with 1 drop of mineral oil (Sigma, Deisenhofen, Germany).

The optimal annealing temperature for each of the 2 primer pairs (Table 2) was determined by evaluating primer sensitivity and specificity across a range of annealing temperatures. Cycling was carried out using a MWG thermal cycler (MWG-Biotech, Ebersberg, Germany). 35 amplification cycles were performed consisting of denaturation at 94 °C for 30 sec, annealing at the optimized temperature for each primer pair 30 sec and extension at 72 °C for 1 min. For the second round of this nested PCR 1 µl from the first PCR reaction were amplified with the inner pair of primers.

Determination of specificity and sensitivity of the EHV-2 specific nested PCR

The amplification products were analysed by electrophoresis in a 2% agarose gel and detected by staining with ethidium bromide. After the first round and second round of the PCR, respectively, a 1 237 bp and a 621 bp fragment were produced. The identity of each of these products was verified by Southern blotting from agarose gels to cellulose nitrate and hybridization with a digoxigenin labeled viral DNA probe. In some cases the restriction enzyme *Dra*I, which cuts the amplificate of the second round once producing 202 bp and 419 bp fragments, was used to provide additional verification of the products.

The sensitivity of the nested PCR was evaluated by serial ten-fold dilutions of EHV-2 DNA from 9 pg to 0.9 fg DNA. After the second round of PCR 0.9 fg (2 genome equivalents) of DNA produced a band in the agarose gel that was clearly visible (Fig. 1).

Southern blot and digoxigenin labeling

Viral DNA extraction and subsequent digestion of DNA was performed as described below. The DNA was separated in 2% agarose gels in Tris-borate-EDTA (TBE; 90 mM Tris-borate, 1 mM EDTA). Electrophoresis was carried out at 5 V/cm. After depurination with 0.4 M HCl for 15 min the denaturation was done in 1.5 M NaCl and 0.5 M NaOH for

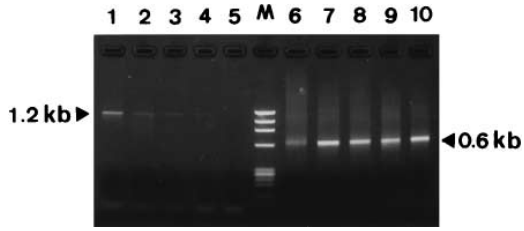

Fig. 1. To determine the detection limit for EHV-2, serial tenfold dilutions of CsCl gradient purified genomic EHV-2 DNA were analysed by nested PCR. 9 pg (*1*), 0.9 pg (*2*), 0.09 pg (*3*), 0.009 pg (*4*) and 0.0009 pg DNA (*5*) were amplified using the outer primer pair, resulting in a 1.2 kb fragment. 0.009 pg of DNA produced a visible band in the 2% ethidium bromide-stained agarose gel (*4*). One μl aliquots of the first PCR reaction products were used as templates for the inner pair of primers, producing a 0.6 kb fragment (*6–10*). The second round of PCR increased the sensitivity of the nested PCR and as little as 0.9 fg of DNA (2 genome equivalents) could be detected in the agarose gel (*10*). DNA markers ϕX-174-*Hae* III and λ-RF/*Hind*III are seen in *M*

additional 15 min. DNA transfer onto a nylon membrane (Boehringer Mannheim) was performed using the VacuGene XL blotting system (Pharmacia) at a stable vacuum of 45 mbar and 1 M Ammonium acetate as transfer buffer. The DNA was linked to the nylon membrane at 120 000 microjoules/cm^2 using a UV crosslinker (Stratagene). Probe labeling was performed by using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. The hybridization was carried out at 42 °C for 20 h using the digoxigenin labeled viral DNA probe at a concentration of 50 ng/ml, in standard hybridization solution containing 50% formamid [25]. Colorimetric detection of the hybridized PCR products with the substrates NBT and BClP (Boehringer Mannheim) was performed according to the manufacturer's protocol.

Purification of PCR amplificates

For restriction enzyme digests PCR products were purified by using the Jetpure Kit (Genomed, Bad Oeynhausen, Germany).

Restriction endonuclease analysis

Products of the second round of PCR were digested with *Dra*I and viral DNA with *Eco*RI and *Hind*III. The fragments generated were separated by electrophoresis through 2% agarose gels and stained with ethidium bromide.

Fragment sequencing

For DNA sequencing, the amplified PCR products were purified as described above. DNA sequence analysis was performed by the dideoxy chain termination method [26] on PCR products as templates by using the ABI PRISM^{TM} dye terminator cycle sequencing ready reaction kit (Perkin Elmer). All steps were carried out as described in the manual. Sequencing products were electrophoretically separated on an ABI 373a automated sequencer. Sequences obtained were compiled using the Sequence navigator software (Applied Biosystem) and the University of Wisconsin Genetics Computer Group software package [10].

Indirect immunofluorescence assay (IFA)

96-well plates seeded with ED cells were infected with 10^{-3} PFU EHV-2 per cell. Cells were fixed with 3% Formalin/phosphate buffered saline (PBS) when CPE was seen. After washing in PBS, cells were permeabilized with 1% Triton X-100 and successively incubated with serial two-fold dilutions of the respective serum and subsequently with the second antibody (fluorescein-conjugated anti-horse IgG, Dianova/Immunotech). Unbound antibody was washed away with 1% NCS in PBS at every step. Cells were examined under a fluorescence microscope (Zeiss, ICM 405). The titre was calculated as the last serum dilution giving a positive antigen staining. Tissue culture plates were coded and red blind.

Results

Detection of infectious virus and viral DNA

To seek a causative agent for the clinical signs observed, we compared numbers of clinically abnormal horses whose PBL samples yielded EHV-2 by co-cultivation and PCR (from groups 1–4) with clinically normal horses (group 5). The highest percentage of positive PCR and cocultivation results (64–71%) were obtained in the group with respiratory tract disease, abortion and ataxia compared to 42% positive animals in the group without clinical symptoms (Table 3).

Cocultivation and PCR results correlated in 36 of the total 106 positive horses, whereas in 52 cases exclusively the PCR and in 18 cases the cocultivation were positive.

Serology

Almost all of the horses were positive for EHV-2-specific antibodies (Table 3) with a range of individual IFA titres from 1:80 to 1:10 240, and most horses (69%) had titres $> 1:640$. Although, some horses from groups 1 and 2 had titres as high as 1:10 000, clinically normal horses (group 5) had titres up to 1:1000. In animals with titres $> 1:2\,560$ a significant correlation with the number of positive PCRs and positive cocultivations was measurable (Table 4) by using the chi-square test ($\zeta = 18.8$).

Nevertheless, in some horses with titres $< 1:640$ virus and viral DNA were detectable and in other horses no virus or viral DNA were detectable, however, they had antibody titres $> 1:640$.

Heterogeneity in the size of PCR products

The products from the second round of the nested PCR showed differences in the size of up to 100 bp (Fig. 2a). Sequence analysis of the products derived from laboratory strains T400 and LK4 revealed that the motif (AGA-CAGGGGCCATGCTGGC) was repeated 9 times in the case of strain T400 and 16 times in the case of strain LK4. Furthermore, in strain LK4, at position 8, a T replaced the G. In T400 there was the nucleotide pair AG present at the beginning of the repeated region, which was absent from strain LK4. All the

Table 3. Results of IFA, PCR and cocultivation

No. of horses belonging to a stud		IFA titre[a]	No. of positive PBL			
			PCR/Cocultivation[b]	PCR[c]	Cocultivation[d]	Total
Group 1:	14	2 560	4	2	3	9
	10	5 120	1	5	1	7
	7	2 560	1	3	0	4
	9	2 560	2	1	2	5
	14	1 280	6	3	2	11
	10	640	3	5	0	8
	5	2 560	2	1	2	5
	4	1 280	2	2	0	4
	3[e]	640	0	0	0	0
Total	76		21 (28%)	22 (29%)	10 (13%)	53 (70%)
Group 2a:	3	5 120	0	3	0	3
	3	2 560	1	1	0	2
	5[e]	2 560	1	1	0	2
Total	11		2 (18%)	5 (45%)	0 (0%)	7 (64%)
Group 2b:	14	1 280	0	7	1	8
	5	2 560	1	1	0	2
Total:	19		1 (5%)	8 (42%)	1 (5%)	10 (53%)
Group 3a:	17[e]	3 840	4	6	2	12
Total:	17		4 (24%)	6 (35%)	2 (12%)	12 (71%)
Group 3b:	11[e]	960	2	3	1	6
Total	11		2 (18%)	3 (27%)	1 (9%)	6 (55%)
Group 4:	19[e]	2560	3	6	1	10
Total:	19		3 (16%)	3 (32%)	1 (5%)	10 (53%)
Group 5:	7	640	3	1	0	4
	11	640	0	1	3	4
	1[e]	640	0	0	0	0
Total:	19		3 (16%)	2 (11%)	3 (16%)	8 (42%)
Total:	172		36 (21%)	52 (30%)	18 (10%)	106 (62%)

[a]IFA titres represent the reciprocal median titres
[b]Number of PBL, positive by PCR and cocultivation
[c]PCR positive only
[d]Cocultivation positive only
[e]Number of individual horses, not belonging to a stud

Table 4. Correlation of IFA titres and the percentages of animals positive by PCR and cocultivation, respectively

IFA titre	$\leq$1:640	1:1280	1:2560	1:5120	1:10240
PCR	49%	46%	30%	82%	86%
Cocultivation	17%	34%	19%	50%	47%

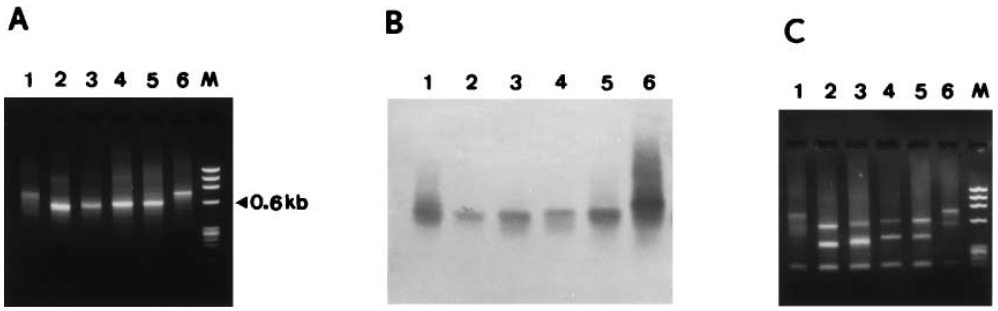

Fig. 2. DNA was extracted from equine PBL of a horse with chronic back problems (*1*), with ataxia (*2*), with abortion (*3*) and with respiratory tract disease (*4*), respectively. 1 µg of it was used as template for the EHV-2 specific nested PCR. Genomic DNA from EHV-2 strains T400 (*5*) and LK4 (*6*) were used as positive controls. The amplificates from the second round of PCR were analysed by gel electrophoresis (**A**) and showed up to 100 bp differences in the molecular weight. For further specification the fragments were hybridized with a digoxigenin labeled EHV-2 genomic DNA probe (**B**) and digested with *Dra*I (**C**), respectively. DNA markers ϕX-174-*Hae*III and λ-RF/*Hind* III are seen in *M*

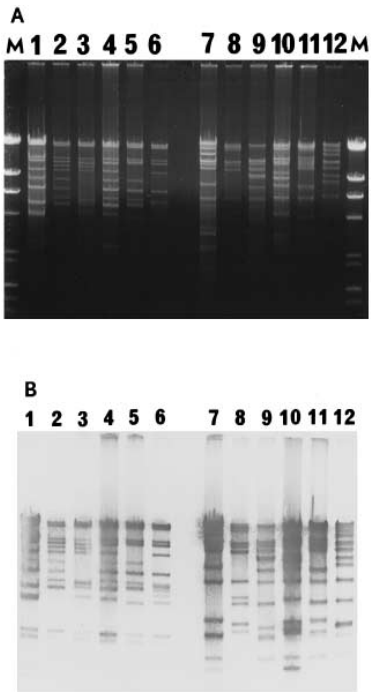

Fig. 3. EHV-2 was isolated by cocultivation from equine PBL of a horse with chronic back problems (*1*, *7*), with ataxia (*2*, *8*), with abortion (*3*, *9*) and with respiratory tract disease (*4*, *10*), respectively. Viral DNA was purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients. Genomic DNA from these isolates as well as from EHV-2 strains T400 (*5*, *11*) and LK4 (*6*, *12*) were digested with *Eco*RI (*1–6*) and *Hind*III (*7–12*), respectively, and analysed by gel electrophoresis (**A**) and Southern blotting (**B**). For hybridization a digoxigenin labeled EHV-2 genomic DNA probe was used. DNA markers ϕX174-*Hae* III and λ-RF/*Hind* III are seen in *M*

PBL nested PCR products hybridized to an EHV-2 specific genome probe (Fig. 2b), suggesting that they were all EHV-2 specific. Restriction digest of the products with the enzyme *Dra*I (Fig. 2c) produced the predicted restriction pattern. The restriction digestion patterns of DNA derived from some viruses isolated from PBL by cocultivation resembled published patterns for EHV-2 [4, 5] (Fig. 3).

Discussion

In this study a nested PCR detected EHV-2 DNA in equine PBL and revealed differences in size of the products of the second round of this PCR of up to 100 bp. Restriction enzyme digests of purified DNA derived from different EHV-2 isolates showed EHV-2 restriction pattern with typical restriction fragment polymorphism. Such genomic heterogeneity is described for EHV-2 [7] and for Human Cytomegalovirus (HCMV) [9]. In both viruses the cleavage site variability is distributed throughout the genome, including the repetitive regions. In HCMV genome variability was also found by PCR and located in the glycoprotein B (gB) gene [9]. However, in an EHV-2 PCR amplifying the gB gene no product polymorphism was observed [21]. The region of the *Eco*RI-N-fragment used in EHV-2 PCR employed in this paper represents a non-coding sequence upstream of the protein homologous to the human and mice Interleukin-10 which is also present in Epstein Barr Virus [22, 29, 35]. Sequencing of the PCR products derived from this genomic region, showed that a motif (AGACAGGGGCCATGCTGGC) was repeated 9–16 times depending on the isolate. Although it has been suggested that genomic variability may play a role in the pathogenicity of EHV-2 isolates [4], there did not appear to be any association between genome variation and pathogenicity in this study. Studies are in progress to define the function of these repeats and their relevance as a tool to differentiate EHV-2 isolates by nested PCR product size.

In the present report we investigated PBL and serum samples from a relatively large number of horses from the Berlin area for evidence of EHV-2 infection using serology, cocultivation of PBL and PCR of DNA derived from PBL. Serum antibodies were detected by IFA in nearly all horses, including clinically normal horses, confirming previous reports [2, 6], that EHV-2 is widespread in the horse population. In contrast, PBL samples were cocultivation positive in only 31% of the horses, but 51% of horses had PCR positive PBL samples. However, PBL samples from 18 horses were positive by cocultivation but negative by PCR. This might be because a relatively small amount of PBL DNA can be used as template in the PCR reaction compared to the number of cells used for cocultivation. Furthermore, by cocultivation latent virus might be reactivated and this amplification step enhances the chance of virus detection. Similar experiences are described by others [21].

Groups 1, 2 and 5 comprised mainly animals from studs. Not unexpectedly, in the group with respiratory tract disease (Group 1) and abortion (Group 2a)

median antibody titres were much higher (1:640–1:5 120) and up to 64% of the animals were PCR positive compared to titres of 1:640 and 27% PCR positive animals in the group without clinical signs (Group 5). From these results one might conclude that among stud horses with an acute infection the distribution of EHV-2 is greater than among animals without symptoms. The relatively high percentage of PCR positive animals in group 5 confirmed the ubiquitous nature of EHV-2. The finding that in some horses no correlation between antibody titres and cocultivation/PCR results were found, might be explained on the one hand with an infection, which has just happened and the antibody production had not been initiated. On the other hand we observed that PBL samples from 6 horses, which were monitored over a period of 6 months by PCR, were initially PCR positive but became PCR negative over the subsequent 1–3 months (data not shown), while low antibody titres were still present.

It has been suggested that PBL, principally B-lymphocytes and/or macrophages, are the site of latent infections [11, 12]. However, from the present study we cannot exclude the possibility that latency may occur in tissues other than the PBL. Thus, we detected EHV-2 by PCR also in the trigeminal ganglia and the olfactory lobe of a foal (unpubl. data) and Edington et al. isolated EHV-2 by cocultivation from trigeminal ganglia from 4 out of 40 abbattoir horses [13]. Studies on experimentally-infected ponies are in progress to extend these findings and to investigate further the role of EHV-2 in clinical disease.

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