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Detection of equine herpesvirus types 2 and 5 (EHV-2 and EHV-5) in Przewalski's wild horses

Brief Report

K. Borchers[1], **K. Frölich**[2], and **H. Ludwig**[1]

[1]Institut für Virologie, FU Berlin, Berlin, Federal Republic of Germany
[2]Institut für Zoo- und Wildtierforschung, Berlin, Federal Republic of Germany

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Summary. In blood samples of seven captive equid species from four German zoos EHV-1 specific antibodies were detected in 76% and EHV-4 specific antibodies in 73% of the 55 animals, whereas 93% were tested positive for EHV-2 and EHV-5, respectively. In only one blood sample from a Przewalski's wild horse EHV-4 DNA was amplified by PCR. From seven Przewalski's wild horses EHV-2, and from another one EHV-5 was isolated by cocultivation. The identity of the virus isolates was verified by PCR and restriction enzyme digestion.

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In equids eight herpesviruses have been identified so far: five Alpha- and three Gamma-Herpesvirinae. The horse is the natural host of equine herpesvirus type 1 (equine abortion virus, EHV-1), equine herpesvirus type 3 (equine coital exanthema virus, EHV-3), and of equine herpesvirus type 4 (rhinopneumonitis virus, EHV-4), which are classified as Alpha-Herpesvirinae [22]. In donkeys an EHV-1- (asinine herpesvirus type 3, AHV-3) and an EHV-3-homologue (asinine herpesvirus type 1, AHV-1) have been described [10]. EHV-2 and the related EHV-5 are Gamma-Herpesvirinae of horses [23], whereas asinine herpesvirus type 2 (AHV-2) represents a related virus of donkeys, which was recovered from leukocytes of a clinically symptom-free animal [9, 11].

EHV-1 and EHV-4 are considered to be the most important viruses in domestic equids [13] causing respiratory disease, abortion, neonatal foal disease, and, more rarely, paresis or paralysis [3, 16, 18]. EHV-2 is widespread throughout the equine population [6, 21] and has been implicated in upper respiratory tract disease, pyrexia, inappetence, lymphadenopathy, immuno-suppression, general malaise, kerato conjunctivitis, and poor performance [6, 8, 19, 24]. EHV-5 shares several

common proteins with EHV-2, however has a different glycoprotein profile [2]. The genome structures of EHV-2 and EHV-5 differ from each other and can be unequivocally distinguished by restriction enzyme analysis [1]. Little is known about the distribution and natural genesis of EHV-5. Only a few EHV-5 isolates have been identified so far. They were isolated from nasal cavities of horses with respiratory disease and from buffy coat cells of horses from Australia and Great Britain [9], however never from wild or zoo equids.

Infections with EHV-1 have been documented in various zoo equids. Thus, in 1992 the neurological form of EHV-1 was diagnosed in 12 animals from six different species at the Berlin Zoo (Przewalski's wild horse, *Equus przewalskii*; Damara zebra, *Equus quagga antiquorum*; Grant's zebra, *Equus quagga boehmi*; Burchell's zebra, *Equus zebra burchelli*; Persian wild ass, *Equus hemionus onager*; domestic ass, *Equus asinus asinus*) [14]. Furthermore, in captive equids from two North American zoos (Lincoln Park Zoo, Chicago and National Zoological Park, Washington DC), EHV-1 was diagnosed by serology, clinical signs and DNA restriction patterns [17, 25]. These studies showed that EHV-1 related isolates from zebras differ from domestic horse EHV-1 strains by antigenic and genomic profiles.

Because an EHV-1 induced abortion has been reported in one Konik mare (*Equus caballus syn ferus*) from the Tiergarten Nürnberg [15], the question arose as to whether besides EHV-1 the other EHV-types might be present in zoo equids and are playing a role in the pathogenesis of abortions or other diseases. These considerations led to the following study on 55 blood samples of equids from different zoos in Germany and the screening for antibodies, viral DNA and infectious virus in these samples.

Blood samples were collected from seven different captive equid species from July 1996 to November 1997 housed in four German zoos (Table 1). The animals were immobilised due to different routine treatments. The blood samples (citrated-buffered) were processed not later than 1–4 days after being taken.

Table 1. Description of the test groups

Group	Species	Zoo Berlin (ZB)	Tierpark Berlin (TB)	Zoo Köln (Kö)	Zoo München (M)	Total
1	Mountain zebra (M)	1	5	–	3	9
2	Chapman's zebra (C)	–	1	–	–	1
3	Grevy's zebra (G)	1	–	1	–	2
4	Przewalski's wild horse (P)	8	1	18	10	37
5	Somali wild ass (S)	–	1	–	–	1
6	Kulan (K)	–	2	–	–	2
7	Kiang (Ki)	–	1	–	2	3
Total		10	11	19	15	55

For measuring neutralising antibodies serial two-fold dilutions of complement-inactivated plasma or serum were incubated with 100 plaque forming units (PFU) of EHV/100 μl for 1 h at 37 °C. After addition of 1×10^5 permissive cells/200 μl/well incubation was continued for 1 h in Eagles's minimum essential medium, Dulbecco's modification (EDM) with 5% new born calf serum (NCS) (Life Technologie GmbH, Berlin, Germany). Cells were finally overlayed with 1.6% carboxymethylcellulose and 2% NCS in EDM. The reaction was stopped with 4% formalin after 2 days (in case of EHV-1 and EHV-4 on equine dermal cells), 7 days (in case of EHV-2 on primary rabbit brain cells) and after 10 days (in case of EHV-5 on primary rabbit spleen cells), respectively. The neutralisation titre was calculated and expressed as the reciprocal of that dilution which produced 50% plaque reduction. The following equine herpesvirus strains were used in the neutralisation test (NT): EHV-1 (MAR-87), EHV-2 (LK4 and T400), EHV-4 (T252) (the strains were kindly supplied by P. Thein, Munich, Germany or were derived from the Institute collection) and EHV-5 (2–141; was kindly provided by M. J. Studdert, Melbourne, Australia). Because neutralising antibodies are in general short lasting, we used in addition the indirect immunofluorescence assay (IFA) to detect EHV type-specific antibodies. This technique has been described elsewhere [6]. The titre was calculated from the highest diluted serum giving a positive antigen staining.

EHV-1 and -4 specific antibody titres were present in 76% and 73% of 55 blood samples from zoo equids, respectively (Table 2). 33% of the zebras had relatively high antibody titres (IFA>1:1280 and NT >1:10) against EHV-1, whereas 8% of the animals had high titres against EHV-4. In comparison, only 5% of the Przewalski's wild horses had high EHV-1 and EHV-4 titres. Among the tested equids, 93% were EHV-2 and EHV-5 sero-positive, respectively. In 33% of all zebras from three different species high EHV-2 antibody titres and in 8% high EHV-5 titres were detectable. In contrast, 73% of the Przewalski's wild horses had high EHV-2 and 27% high EHV-5 titres. In kiangs and the Somali wild ass we measured only low EHV-1 and EHV-4 specific antibody titres by IFA. EHV-2 or EHV-5 neutralising antibodies were not detectable, however by IFA all kulans, kiangs and the Somali wild ass had titres against EHV-2 and EHV-5 in the range of 1:160–1:2560.

EHV-1-, EHV-2- and EHV-4-infections are widespread and well known in domestic horses, whereas only a few reports exist on zoo and wild equids [14, 15, 17]. In free-ranging zebras in Southern Africa, Barnard and Paweska [4] described a high antibody prevalence against EHV-1 and EHV-4 in Burchell's zebras (*Equus zebra burchelli*) in the Kruger National Park, suggesting both herpesvirus infections being enzootic. Recently, we found EHV-1, EHV-2 and EHV-4 antibodies in free-ranging mountain zebras (*Equus zebra*) from Namibia, whereas EHV-3 and EHV-5 specific antibodies were not detectable in the tested animals [7]. In our present study on equids from four German zoos, 73–76% of the animals had EHV-1 and -4 specific antibody titres. Because zebras and Przewalski's wild horses from the Munich Zoo are regularly vaccinated with Prevaccinol (live attenuated EHV-1, RAC-H strain), the titres most probably represent vaccine-induced

Table 2. Results of IFA and NT

Group	animal	EHV-1		EHV-2		EHV-4		EHV-5	
		IFA[a]	NT[a]	IFA	NT	IFA	NT	IFA	NT
1[b]	M/TB 2[c]	80	–[d]	5120	–	–	–	1280	–
	M/TB 7	1280	–	1280	–	320	–	640	–
	M/TB 8	640	–	2560	–	320	–	640	–
	M/TB 9	160	–	1280	–	–	–	1280	–
	M/TB 11	640	–	160	–	1280	–	1280	–
	M/M 19	2560	–	320	–	640	–	160	–
	M/M 20	2560	10	640	–	1280	–	320	–
	M/ZB 23	–	–	640	–	–	–	1280	–
	M/M 38	10240	–	5120	80	640	–	1280	–
2	C/TB 6	1280	–	5120	–	160	–	2560	–
3	G/ZB 22	–	–	320	–	–	–	320	–
	G/Kö 39	5120	–	160	–	2560	–	640	–
4	P/M 3	640	–	10240	–	160	–	2560	40
	P/M 5	1280	–	2560	–	1280	–	1280	–
	P/TB 12	–	–	2560	–	–	–	320	–
	P/Kö 14	160	–	5120	–	–	–	1280	–
	P/ZB 16	80	–	1000	–	20	–	nd[e]	nd
	P/ZB 17	40	–	750	–	20	–	nd	nd
	P/Kö 18	40	–	300	–	20	–	nd	nd
	P/ZB 24	–	–	5120	60	–	–	1280	–
	P/ZB 25	–	–	1280	–	–	–	–	–
	P/ZB 26	–	–	2560	10	–	–	320	nd
	P/Kö 30	160	–	640	–	320	–	640	–
	P/Kö 31	640	–	2560	–	1280	–	640	–
	P/Kö 32	160	–	5120	–	160	–	640	–
	P/Kö 33	640	–	5120	20	640	–	2560	20
	P/Kö 34	160	–	10240	20	160	–	640	20
	P/Kö 35	320	–	10240	–	640	–	1280	–
	P/Kö 36	–	–	10240	–	160	–	5120	20
	P/Kö 37	40	–	10240	–	160	–	2560	–
	P/M 40	640	–	640	40	640	–	1280	–
	P/M 41	–	–	1280	20	–	–	1280	–
	P/M 42	–	–	5120	80	–	–	320	–
	P/M 44	320	–	20480	60	640	10	5120	–
	P/M 45	320	–	10240	60	160	–	2560	–
	P/M 46	320	–	2560	–	80	10	640	10
	P/M 47	320	–	20480	80	320	–	2560	–
	P/M 48	5120	20	640	–	1280	10	2560	40
	P/ZB 49	40	–	–	–	1280	40	640	–
	P/ZB 50	320	–	–	–	640	–	1280	–
	P/ZB 51	80	–	–	–	640	–	640	–
	P/Kö 53	640	–	2560	40	160	–	320	–
	P/Kö 54	640	–	1280	20	40	–	320	–
	P/Kö 55	640	–	5120	20	40	–	2560	–

Table 2 (*continued*)

	P/Kö 56	1280	–	5120	20	160	–	640	–
	P/Kö 57	640	–	1280	20	160	–	160	–
	P/Kö 58	640	–	1280	–	40	–	160	–
	P/Kö 59	5120	40	2560	10	2560	–	640	–
	P/Kö 60	320	–	5120	10	40	–	160	–
5	S/TB 10	160	–	160	–	160	–	1280	–
6	K/TB 28	–	–	2560	–	–	–	2560	–
	K/TB 29	–	–	2560	–	–	–	1280	–
7	Ki/M 43	–	–	–	–	–	–	2560	–
	Ki/TB 52	320	–	320	–	–	–	1280	–
	Ki/M 61	–	–	1280	–	320	–	2560	nd

[a]Titres represent the reciprocal titres
[b]The discription of the groups are presented in Table 1
[c]Abbrevations for the species and zoos are given in Table 1
[d]Titres <1:10 in IFA and NT were regarded as negative
[e]Not done

antibodies, whereas the other animals were not vaccinated and therefore seem to suffer from natural infections. In three animals (No. 38, 48, 59) the height of the EHV-1 IFA titre (≥1:5120) and/or the increased NT titres (≥1:20) indicated an acute infection or reactivation, although only Przewalski's wild horse No. 59 showed clinical signs, such as inappetence, muscle tremors, fever and apathy. In Przewalski's wild horse No. 49 did the EHV-4 NT titres suggest an acute infection; the virus itself however, was not detectable.

Recently, we reported EHV-2 specific antibody titres in 95% of free-living mountain zebras from Namibia. Viral DNA, however, could not be amplified [7]. In 24% of the zoo equids investigated here, the EHV-2 IFA and/or NT titres were significantly increased, nevertheless none of the animals showed clinical signs. Based on our experience with domestic horses, EHV-2 IFA titres higher than 1:5120 and NT titres higher than 1:20 are regarded as conspicious.

Epidemiological data on the incidence of EHV-5 infections in horses are not available. Because EHV-2 and EHV-5 are antigenically related an unequivocal serological differentiation is difficult (for references see [20]). Accordingly, in our present study almost all of the EHV-2 sero-positive animals had also EHV-5 antibodies, with the exception of Przewalski's wild horse No. 25, in which EHV-2, however no EHV-5 antibody titres were found. In addition, four animals (No. 43, 49, 50, 51) had antibodies against EHV-5, yet no antibody titres against EHV-2 were measured. In another Przewalski's wild horse (No. 48) with relatively high EHV-5 IFA and NT titres, comparatively low EHV-2 specific antibodies were detectable. This suggests a more or less strong serological cross-reactivity, probably depending on the EHV-2 and/or EHV-5 strain.

Peripheral blood leukocytes (PBL) were yielded from venous blood supplemented with 1/10 volume of sodium-citrate (3 g/100 ml PBS) by centrifugation through a Ficoll gradient. From ten animals (Table 3), 2–5 $\times$ 10^6 PBL were seeded

Table 3. Results of PCR and cocultivation

Group	Animal	PCR				Cocultivation
		EHV-1	EHV-2	EHV-4	EHV-5	
1[a]	M/TB 2[b]	–[c]	–	–	–	nd[d]
	M/TB 7	–	–	–	–	nd
	M/TB 8	–	–	–	–	nd
	M/TB 9	–	–	–	–	nd
	M/TB 11	–	–	–		nd
	M/M 19	–	–	–	–	nd
	M/M 20	nd	–	–	–	nd
	M/ZB 23	nd	nd	nd	nd	nd
	M/M 38	–	–	–	–	nd
2	C/TB 6	–	–	–	–	nd
3	G/ZB 22	nd	nd	nd	nd	nd
	G/Kö 39	–	–	–	–	nd
4	P/M 3	–	–	–	–	nd
	P/M 5	–	–	–	–	nd
	P/TB 12	–	+	–	–	nd
	P/Kö 14	–	–	–	nd	nd
	P/ZB 16	–	–	–	–	nd
	P/ZB 17	–	+	–	–	nd
	P/Kö 18	–	+	–	–	nd
	P/ZB 24	nd	nd	nd	nd	nd
	P/ZB 25	nd	nd	nd	nd	nd
	P/ZB 26	nd	nd	nd	nd	nd
	P/Kö 30	–	–	–	–	nd
	P/Kö 31	–	–	–	–	nd
	P/Kö 32	–	–	–	–	nd
	P/Kö 33	–	–	–	–	nd
	P/Kö 34	–	+	–	–	nd
	P/Kö 35	–	–	–	–	EHV-2+
	P/Kö 36	–	+	–	–	EHV-2+
	P/Kö 37	–	+	–	–	EHV-2+
	P/M 40	–	–	+	–	–
	P/M 41	–	+	–	–	nd
	P/M 42	–	+	–	–	nd
	P/M 44	–	+	–	nd	EHV-2+
	P/M 45	–	+	–	nd	EHV-2+
	P/M 46	–	+	–	nd	EHV-2+
	P/M 47	–	+	–	nd	EHV-2+
	P/M 48	–	–	–	+	EHV-5+
	P/ZB 49	–	+	–	–	nd
	P/ZB 50	–	+	–	–	nd
	P/ZB 51	–	–	–	–	nd
	P/Kö 53	–	–	–	–	nd
	P/Kö 54	–	–	–	–	nd
	P/Kö 55	–	–	–	–	nd

Table 3 (*continued*)

	P/Kö 56	–	–	–	–	nd
	P/Kö 57	–	–	–	–	nd
	P/Kö 58	–	+	–	–	nd
	P/Kö 59	–	–	–	–	nd
	P/Kö 60	–	+	–	–	nd
5	S/TB 60	–	–	–	–	nd
6	K/TB 28	–	–	–	nd	nd
	K/TB 29	–	–	–	nd	nd
7	Ki/M 43	–	–	–	–	–
	Ki/TB 52	–	–	–	–	nd
	Ki/M 61	–	nd	–	nd	nd

[a]The description of the groups are presented in Table 1
[b]Abbreviations for the species and zoos are given in Table 1
[c]PCR negative
[d]Not done

into tissue culture plates (Nunc) together with an equal number of permissive cells for cocultivation (for details see [6]). Virus from supernatants was pelleted by ultra centrifugation through a 20% succrose cushion, 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-gradients. Purified EHV-2 and EHV-5 DNA (200 ng) were digested with the restriction enzymes *Eco* RI and *Hind* III, respectively. The generated fragments were separated by electrophoresis through a 1% agarose gel and stained with ethidium bromide.

For DNA extraction 10^4–10^6 PBL of each animal were treated as described elsewhere [6] and analysed by type-specific nested PCRs [5, 6]. The PCR mix (50 μl) contained 10 pg of purified viral DNA or 1 μg of cellular DNA. Cycling was carried out using a MWG thermal cycler (MWG-Biotech, Ebersberg, Germany). Thirty-five amplification cycles were performed consisting of denaturation at 94 °C for 30 sec, annealing at the optimized temperature for each primer pair for 30 sec and extension at 72 °C for 1 min. For the second round of this nested PCRs, 1 μl from the first PCR reaction was amplified with the nested pair of primers. The sensitivity of the nested PCRs was evaluated by serial ten-fold dilutions of EHV DNA from 10 pg to 1 fg of DNA. After the second round of PCR, 1–2 genome equivalents of DNA produced a visible band in the agarose gel.

For amplification of DNA from the EHV-5 gB gene, we used a primer pair described by Agius et al. [2] under conditions optimized on a MWG thermal cycler. One amplification cyle consisted of the following three steps, which were repeated 38 times: denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min. The amplification products were 1.3 kb in length.

All buffy coat samples tested negative for EHV-1 by either PCR or coculti-vation. EHV-4 DNA was detected in one Przewalski's wild horse No. 40 with unsuspicious antibody titres (Table 3). Interestingly, EHV-2 DNA were amplified

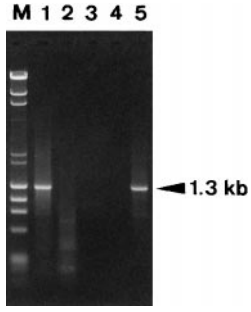

Fig. 1. PBL of Przewalski's wild horse No. 48 were cocultivated on primary rabbit spleen cells. After the appearance of cytopathic effects, cells were harvested and DNA were extracted. 1 μg of it was used as template for the EHV-5 specific PCR (*1*). DNA from non-infected primary rabbit spleen cells (*2*), genomic DNA from EHV-2 strain LK4 (*3*) and EHV-5 strain 2–141 (*5*) were used as controls. *4* contains a negative reagent control. The amplification products were analysed by gel electrophoresis. DNA markers ϕX-174-*Hae* III and λ-RF/*Hind* III are seen in position *M*

from 16 Przewalski's wild horses. From seven of them EHV-2 could also be isolated. In nine Przewalski's wild horses the height of the titres correlates with a positive PCR and/or virus isolation result (No. 34, 35, 36, 37, 41, 42, 44, 45 and 47), suggesting an acute infection or reactivation.

Viral DNA from EHV-2 isolates of Przewalski's wild horses No. 35, 37, 44–47 were specified by nested PCR. The amplification products of the second round showed minor molecular weight differences in isolates No. 37 and 45 in comparison to the reference strain (data not presented). As known from previous studies [6], this indicates genomic strain variations as typical of EHV-2 [12].

EHV-5 DNA were amplified from the PBL of Przewalski's wild horse No. 48, which had high EHV-5 NT and IFA titres, however no clinical signs were observed. Because it was not a nested PCR, we further specified the amplification products by Southern-hybridization. The PCR products derived from the PBL of animal No. 48 hybridized to the Digoxigenin-labeled EHV-5 genomic probe, confirming the EHV-5 specificity (data not presented). Furthermore, we isolated a slow growing virus from the PBL of the same animal by cocultivation on primary rabbit spleen cells. By PCR we amplified EHV-5 specific sequences as shown in Fig. 1. In order to further define the new isolate (EHV-5.KB-P48), purified viral DNA were digested with *Eco* RI and *Hind* III, respectively. DNA from EHV-2 strain LK4 as well as from strain EHV-5.2–141 were used as references. In general, the restriction enzyme profile of EHV-5.KB-P48 resembled patterns of EHV-5. 2–141, with a striking difference in the *Eco* RI fragments C and D (Fig. 2). Thus in case of isolate EHV-5.2–141 these fragments had a molecular weight of 6.7 kb and 6.5 kb (Fig. 2, lane 2), as described by Agius et al. [1], whereas the respective fragments generated from EHV-5.KB-P48, were about 7 kb and 6 kb in length (Fig. 2, lane 1). These findings suggest strain specific sequence variations, resulting in the position change of the *Eco* RI cleavage site.

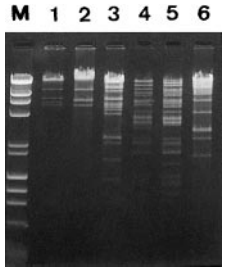

Fig. 2. Virus was isolated by cocultivation of the PBL from Przewalski's wild horse No. 48 and was used for DNA extraction. 200 ng of the CsCl-gradient purified viral DNA of the isolate EHV-5.KB-P48 (*1* and *4*), from reference strain EHV-5.2–141 (*2* and *5*) as well as from EHV-2 reference strain LK4 (*3* and *6*) were digested with *Eco* RI (*1–3*) and *Hind* III (*4–6*), respectively, and analysed by gel electrophoresis. DNA markers ϕX-174-*Hae* III and λ-RF/*Hind* III are seen in position *M*

From these results we conclude, that EHV-5 is present in Przewalski's wild horses. Further studies on the prevalence of EHV-5 infections in domestic horses are in progress. Moreover, studies are necessary to investigate the role of EHV-2 and EHV-5 in the pathogenesis and possibly reactivation of EHV-1 and EHV-4. Because few informations exist such studies build the basis for estimating and controlling herpesvirus outbreaks among zoo and wild equids and are finally the presupposition for diagnostic and vaccination programs.

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Authors' address: Dr. K. Borchers, Institut für Virologie, FU Berlin, Königin-Luise-Str. 49, D-14195 Berlin, Federal Republic of Germany

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