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Source: Journal of Wildlife Diseases, 33(4) : 812-817

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-33.4.812

Journal of Wildlife Diseases, 33(4), 1997, pp. 812–817

ANTIBODIES AGAINST EQUINE HERPESVIRUSES IN FREE-RANGING MOUNTAIN ZEBRAS FROM NAMIBIA

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ABSTRACT: Twenty-one blood samples of free-ranging mountain zebras (*Equus zebra*) from Namibia were tested for equine herpesvirus (EHV-1, -2, -3, -4) specific antibodies by immunofluorescence assay (IFA) and neutralization test (NT). Additionally, type-specific nested polymerase chain reactions (nested PCR) were employed for detection of EHV-1, -2 and -4 DNA. Equine herpesvirsus-1 antibodies were detected by IFA in all zebras, while only seven serum samples contained EHV-4 IFA antibodies. Sera with high IFA antibodies also were found to neutralize EHV-1 and -4. Furthermore, 20 zebras were EHV-2 seropositive by IFA, and one zebra had EHV-2 neutralizing antibodies. Equine herpesvirus-3 specific antibodies were not detected. We did not amplify EHV-1, -2 or -4 specific DNA sequences in peripheral blood leukocytes of the same zebras using type-specific nested PCR. EHV infections appear to be widespread in free-ranging zebras, as they are in domestic horses.

Key words: Equine herpesviruses, 1–4, *Equus zebra*, mountain zebra, polymerase chain reaction, serologic assay.

INTRODUCTION

The horse is natural host of five herpesviruses; three are classified as Alphaherpesvirinae and two as Gammaherpesvirinae. The Alphaherpesvirinae have a variable host range, they have short replicative cycles, and quickly induce lysis of infected cells (Roizman, 1996). Furthermore, all become latent primarily, but not exclusively, in nervous tissues. The three equine Alphaherpesviruses are equine herpesvirus type 1 (equine abortion virus, EHV-1), equine herpesvirus type 3 (equine coital exanthema virus, EHV-3), and equine herpesvirus type 4 (rhinopneumonitis virus, EHV-4). Equine herpesvirus-1 and EHV-4 are considered to be the most important and widespread viruses (Crabb and Studdert, 1995).

Equine herpesvirus-1 infection is the cause of four distinct syndromes in horses: respiratory disease (rhinopneumonitis), abortion, neonatal foal disease, and, more rarely, paresis or paralysis (Allen and Bryans, 1986; Mumford, 1994). Furthermore, immunity is short-lived and horses experience repeated infections throughout life as a result of re-exposure (Thomson et al., 1976) or reactivation of latent virus (Burrows and Goodridge, 1984). In older animals, these infections are often subclinical (Mumford, 1994). Transmission occurs by direct or indirect contact with infective nasal discharges, aborted fetuses, and placentas (Schneider, 1994). Equine herpesvirus-4 predominantly causes respiratory disease (Studdert, 1974) which is indistinguishable from that caused by EHV-1, and most horses are infected during the first 2 yr of life.

Equine herpesvirus type 2 (EHV-2) and the related equine herpesvirus type 5 (EHV-5) are Gammaherpesviruses (Telford et al., 1993). Equine herpesvirus-2 is thought to be widespread in the equine population (Roeder and Scott, 1975). Although EHV-2 has been implicated in upper respiratory tract disease, pyrexia, inappetence, lymphadenopathy, immunosuppression, general malaise, keratoconjunctivitis, and poor performance (Pálfi et al., 1978; Thein and Böhm, 1976), its role as an equine pathogen remains uncertain. Furthermore, EHV-2 has been isolated from tissues of apparently healthy horses and almost all adult horses surveyed were seropositive (Browning and Studdert, 1988).

Two alphaherpesviruses, an EHV-1 ho-

mologue (asinine herpesvirus type 3, AHV-3) and an EHV-3 homologue (asinine herpesvirus type 1, AHV-1), were isolated (Browning et al., 1988) from donkeys. Furthermore, an EHV-2 related Gammaherpesvirus, asinine herpesvirus type 2 (AHV-2), was recovered from leukocytes of a clinically normal donkey (Browning and Studdert, 1988).

In South Africa EHV-1, EHV-4, African horsesickness virus (AHSV), equine encephalosis virus (EEV), equine influenza virus A (Equi 2), and equine arteritis virus (EAV) are some of the more important viral pathogens of horses (Barnard and Paweska, 1993). Knowledge of the involvement of free-living zebras in the epidemiology of viral diseases of Equidae is very limited, with the exception of their involvement in AHS (Erasmus et al., 1978; Barnard, 1993). Equine herpesvirus-1 infections in domestic horses were first confirmed in Namibia in 1988, with sporadic occurence since then (Schneider, 1994). There is only one report from Africa of free-ranging zebras with EHV, as demonstrated by the presence of antibodies against EHV-1 and EHV-4 in Burchell's zebras (*Equus burchelli*) from Kruger National Park (KNP) (Barnard and Paweska, 1993). However, EHV-1 has been diagnosed by serology, clinical signs, and DNA restriction patterns in zebras from zoos (Montali et al., 1985; Kahrmann et al., 1993).

The eptizootiology of EHV-infection in free-ranging mountain zebras (*Equus zebra*) and the distribution of these diseases in different zebra populations in Africa is not yet clear. Our objective was to determine whether free-ranging mountain zebras in central Namibia were naturally infected by EHV and therefore might be involved in the natural cycle of these viruses.

MATERIALS AND METHODS

We collected 21 blood samples from free-ranging montain zebras between September and November 1992. All animals were culled and blood was drawn immediately after death. The zebras were selected randomly. Hunters provided blood samples together with information on sex and age. Within 24 hr the cooled blood samples were centrifuged and buffy coat was removed. Sera were stored at −70 C and transported in cooled boxes to the Robert Koch-Institute (Berlin, Germany) in November 1992 and were stored again at −70 C.

The samples originated from private farmland northwest of Windhoek (22°40′S to 22°50′S, 16°20′E to 16°30′E) and from the Hardap Recreation Resort southeast of Windhoek (24°45′S to 24°55′S, 17°60′E to 16°90′E) Namibia. Both areas are in the Central Plateau, which is characterized by rugged mountains and wide, open undulating plains and varies in altitude from 1,000 to 1,600 m. Vegetation is savanna-type and characterized mainly by grasses, shrubs, and trees, with a great variety of species. In both areas permanent water is available in pools. Both natural and artificial water sources are available for wildlife; seasonal waterholes are filled after the rains, while boreholes provide water during the dry period. The climate of the study area is characterized by seasonal temperature variation such that in December the temperature may reach 40 C and in July it falls to −5 C. Mean annual relative humidity is 50% and the mean annual rainfall varies between 15 and 20 mm. In and around the study areas, domestic horses and donkeys roam almost freely. Other species of Perissodactyla are not endemic.

Peripheral blood leukocytes (PBL) were prepared from heparinized venous blood by centrifugation through a Ficoll (Seromed, Berlin, Germany) gradient (density: τ = 1.077 g/ml). For DNA extraction, 1×10^4 to 1×10^6 PBL were resuspended in 50 to 400 µl proteinase K digestion buffer (50 mM Tris pH 8.5; 1mM EDTA; 0.5% Tween 20). After addition of 1µl of proteinase K (10mg/ml; Boehringer, Mannheim, Germany) per 100 µl suspension, cells were incubated for 1 hr 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 4,000 × G and the supernatant used for the polymerase chain reaction (PCR).

In order to detect EHV-2 DNA with high sensitivity and specificity we developed a type-specific nested PCR (Borchers et al., 1997) based on a sequence upstream nof the open reading frame coding for a product homologous to human interleukin-10, which is located in the *Eco*RI N-fragment of the EHV-2 strain T400 (Rode et al., 1993). The outer primer pair was 20 nucleotides long (P1: 5′-GGCACTGAA-ACCCCATACTG-3′; P2: 5′-AAAACCATCCT-GTCCAACCA-3′) and the inner primer pair was 18 nucleotides long (P3: 5′-CCACTAAC-

CCCCAACCTT-3'; P4: 5'-CCTC TATCCT-CACAACAG-3'). 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, New Jersey, USA) and 1× reaction buffer (Perkin Elmer Cetus). The aqueous phase was covered with 1 drop of mineral oil (Sigma, Deisenhofen, Germany). Cycling was carried out using a MWG thermal cycler (MWG-Biotech, Ebersberg, Germany). Amplification cycles were performed 35 times and consisted of denaturation at 94 C for 30 sec, annealing at 64 C for 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. The amplificates were analyzed in ethidium bromide stained 2% agarose gel. After the first round of amplification a 1.2 kb fragment and after the second round a 0.6 kb fragment was produced. The sensitivity of the nested PCR was 0.9 fg (2 genome equivalents) after the second round of PCR.

For detection and differentiation of EHV-1 and EHV-4 DNA, respectively, a nested PCR, consisting of two consecutive PCRs using g-B-specific primers for EHV-1 and EHV-4, was carried out (Borchers and Slater, 1993). The amplificates were analyzed in ethidium bromide stained 1% agarose gel. The detection limit of the EHV-1 PCR is 1 fg (3 genome equivalents) and that of the EHV-4 PCR 0.1 fg (1 genome equivalent).

Serial two-fold dilutions of complement inactivated plasma or serum were incubated with 100 plaque forming units (PFU) of EHV/100 μl for 1 hr at 37 C. After addition of 1×10^5 equine dermal (ED) cells/ 200 μl/ well the incubation was continued in Dulbecco's Modified Eagle Medium (EDM) with 5% new born calf serum (NCS) (Life Technologie GmbH, Berlin, Germany) for 1 hr and finally overlayed with 1.6% carboxymethylcellulose and 2% NCS in EDM. The reaction was stopped in the case of EHV-1 and EHV-4 after 2 days and in the case of EHV-2 after 1 wk with 4% formalin and the plaques were stained with Giemsa. The neutralization titer was calculated and expressed as the reciprocal of the dilution that produced 50% plaque reduction. The following equine herpesvirus strains were used in NT: EHV-1 (MAR), EHV-2 (LK4), and EHV-4 (T252); all strains were kindly supplied by P. Thein (München, Germany).

Indirect immunofluorescence assay (IFA) was conduced on 96-well plates seeded with ED cells were infected with 1×10^{-3} PFU EHV per cell. Cells were fixed with 3% formalin/phosphate buffered saline (PBS) when cytopathic effects were 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, Hamburg, Germany). Unbound antibody was washed away with 1% NCS in PBS at every step. Cells were examined under a fluorescence microscope (ICM 405, Zeiss, Göttingen, Germany). The IFA was calculated as the last serum dilution giving positive fluorescence. Tissue culture plates were coded and read without knowledge of the scource.

RESULTS

Twenty-one zebra sera were tested for antibodies against EHV-1, EHV-2, EHV-3, and EHV-4 (Table 1). By IFA all sera were EHV-1 antibody positive with a range of titers from 1:40 to 1:1,280. By NT three of the sera neutralized EHV-1. Only seven zebras had EHV-4 antibodies (1:80–1:160) by means of IFA and sera with high IFA antibodies also neutralized EHV-4 (1:10–1:20). Twenty sera had high EHV-2 specific IFA antibody titers (1:80–1:1,280) and in one case a NT titer (1:80) against EHV-2. Antibodies against EHV-3 were not detected.

In addition to serology, we extracted DNA from the 21 zebra PBL samples and analyzed them by type specific nested PCRs. None of the samples contained EHV-1, -2 or -4 specific sequences.

DISCUSSION

No clinical signs suggesting EHV infection where observed by the hunters, but all of zebras were seropositive for EHV-1 specific IFA antibodies. However, neutralizing antibodies were found only in three zebras. Lack of clinical signs in seropositive animals is similar to domestic horses, which we recently investigated in the Berlin area of Germany (K. Borchers and U. Wolfinger, unpublished data). In such horses IFA antibody titers higher than 1:1,280 indicate a recent acute infection. Whereas, NT antibodies increase after an acute infection from <1:10 to 1:160 and last in comparison to IFA antibodies

TABLE 1. Reciprocal titers of equine herpesvirus antibodies in 21 free-ranging mountain zebras from Namibia.

Zebra number	IFA[b]			NT[b]		
	EHV-1[a]	EHV-2[a]	EHV-4[a]	EHV-1	EHV-2	EHV-4
1	640	320	—[c]	—	—	—
2	640	640	—	—	—	—
3	40	1,280	—	—	80	—
4	320	1,280	—	—	—	—
5	320	1,280	80	—	—	—
6	1,280	1,280	80	—	—	10
7	640	1,280	—	—	—	—
8	320	1,280	—	—	—	—
9	160	320	—	—	—	—
10	640	1,280	—	—	—	—
11	1,280	320	—	—	—	—
12	1,280	1,280	160	20	—	10
13	1,280	320	80	10	—	20
14	1,280	640	160	20	—	20
15	640	1,280	160	—	—	10
16	640	640	160	—	—	20
17	80	—	—	—	—	—
18	160	1,280	—	—	—	—
19	640	1,280	—	—	—	—
20	80	1,280	—	—	—	—
21	80	1,280	—	—	—	—

[a] EHV-1 = equine herpesvirus type 1; EHV-2 = equine herpesvirus type 2; EHV-4 = equine herpesvirus type 4.
[b] NT = neutralization test; IFA = immunofluorescence assay.
[c] — = negative; titers <1:10 in IFA and NT were regarded as negative.

(months) only for a short period of time (week). The titer limits are based on the specific tests used in our laboratory and furthermore depend on the EHV reference strain. Therefore, titers derived from different laboratories are not comparable. Based on our experience with domestic horses we would interprete the titers measured in the zebras as results of a former infection with EHV-1 and not as an acute one.

The relative low prevalence of EHV-4 antibodies (33%) in the free-ranging zebras is in contrast to our results from horses and that of others from captive zebras. Thus, Barnard and Paweska (1993) reported high antibody prevalence against EHV-1 (87%) and EHV-4 (92%) in Burchell's zebras, suggesting that both EHV infections are endemic among zebras in KNP. Furthermore, titers of antibodies against EHV-4 in domestic horses in Berlin (U. Wolfinger and K. Borchers, unpublished data) and in zebras (Barnard and Paweska, 1993) are often higher than that against EHV-1. In the zebra sera we analyzed, however, the opposite was true. This suggests that the reference strain we used for diagnosis is more closely related to the EHV-1 strain of the zebras or more likely that the results are due to cross-reactivity of the sera between the two closely related viruses.

No Gammaherpesvirus has been described in zebras. Therefore, it was unexpected that 19 of 20 zebras we investigated had relative high antibody titers. The hight of the titers in comparison to that against EHV-1 and EHV-4 makes a cross-reaction of EHV-1 or EHV-4 specific antibodies with EHV-2 very unlikely. Furthermore, with EHV-1 or -4 specific horse sera we have never found a serologic cross-reaction with EHV-2 (K. Borchers, unpublished data).

Although some of the zebras had high

EHV-1, -2 or -4 titers, none contained EHV specific DNA sequences. Especially for EHV-2 it is well known that the genome varies from strain to strain (Browning and Studdert, 1987; 1989). Therefore, the negative PCR results might be due to non-binding of the primers used because of sequence mutations in the zebra virus genome. The same argument might explain why we could not detect EHV-1 or -4 specific sequences in the zebra PBL. From studies of Montali et al. (1985) and Wolff et al. (1986) it is known that EHV-1 related zebra isolates differed from domestic horse EHV-1 strains by antigenic and genomic profiles. Another explanation for lack of detection of herpesviral DNA in blood is that the animals may not have been viraemic in general or at the moment of investigation. For EHV-4 it is known, that infections only seldomly lead to viraemia in domestic horses (Matsumura et al., 1992). The occurence of viraemia following infections with EHV-1 and EHV-2, however, has been reported by several groups (Scott et al., 1983; Drummer et al., 1996; Borchers et al., 1997). However, as known from experimental infections of ponies EHV-1 appears in PBL only for a short period (between day 3 and 15) (Slater et al., 1994).

In conclusion, EHV-1, -2 and -4 infections are probably widespread among free-ranging zebras and seem to be similar in zoo and domestic equids. Further investigations are needed to clarify the origin of these herpesviruses. Since transmission of EHV from donkeys or horses can not be ruled out, the introduction onto game farms should be restricted to animals proven to be free of EHV.

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Received for publication 6 November 1996.