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BRIEF REPORT

Subclinical infection of a young captive Asian elephant with elephant endotheliotropic herpesvirus 1

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Abstract Elephant endotheliotropic herpesviruses (EEHVs) are a continuous threat for young Asian elephants. We report a laboratory-confirmed infection of a 5-year-old female Asian elephant (AZ_2016) in the Berlin Zoologischer Garten. Initially, high EEHV-1 loads were detected in trunk swabs obtained from the young elephant during routine screening. The animal showed no clinical signs except for slight irritability. EEHV-1 was continuously shed for almost one year, with fluctuations in viral load from time to time. Our investigations highlight the continuous threat of EEHV-1 to young captive Asian elephants and stress the importance of routine monitoring of captive elephants to allow early detection of infection.

Keywords EEHV-1 · Asian elephants · Zoo

Elephant endotheliotropic herpesvirus type 1 (EEHV-1) and the other known elephant herpesviruses, EEHV-2 to -7, are members of the family *Herpesviridae* and the genus *Proboscivirus* [3]. The proboscivirus are ubiquitous pathogens that can infect both African (*Loxodonta africana*) and Asian elephants (*Elephas maximus*) and can induce a range of clinical signs [2, 9]. It is documented that EEHV infections in African and adult Asian elephants are usually asymptomatic with occasional virus shedding in trunk secretions. However, in young Asian elephants, the virus can cause acute fatal hemorrhagic disease and is considered a significant threat to elephant calves, both in captivity and in the wild [1, 7, 15, 17, 20, 21]. The clinical signs manifest by a sudden onset and start, initially non-specifically, but regularly develop into hemorrhagic disease, which is caused by widespread endothelial damage and results in death within 1-5 days [10, 11]. Other clinical signs can include anorexia, lethargy, oral ulcerations, purple tongue, and edema in the head, neck and limbs. Although mostly fatal, several reports have documented several juvenile Asian elephants that survived EEHV infections [2, 9, 12]. Establishment of latency after EEHV infection has not been reported, let alone understood; however, latency is considered a characteristic feature common to all herpesviruses [6], and this propensity may explain the periodical shedding of the virus in trunk secretions, while at other times, no virus can be detected in the animals.

The route of transmission of EEHVs to naïve individuals is still unknown. Several reports have indicated that virus shedding in secretions from the trunk, conjunctiva, palate, and vulva could present a possible route of virus transmission to calves after contact with infected materials. On the other hand, there is no evidence of virus shedding in semen. Furthermore, vertical transmission through natural breeding or artificial insemination has not been recorded; however, this route cannot be excluded. Previous reports have shown that EEHVs can be isolated from skin papilloma of apparently healthy elephants, which could provide another route of virus transmission to susceptible elephants [2, 7, 12–15].

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Elephants are red-listed by the International Union for Conservation of Nature (IUCV) as endangered and threatened to become extinct (www.iucnredlist.org). To improve elephant breeding in captivity and to control infectious diseases, several zoological collections have implemented a regular program for monitoring captive Asian and African elephants, particularly calves, as recommended by the EEHV Advisory Group and the World Association of Zoos and Aquariums (AZA) Taxon Advisory Group (TAG).

Although close monitoring and early detection of EEHV infection together with good medical care have helped to save several acutely infected juvenile Asian elephants, the use of antiviral drugs such as famciclovir (FCV) or ganciclovir (GCV) for treatment is still controversial [2, 16].

In an attempt to follow EEHV infection longitudinally, we carried out routine monitoring for EEHV in Asian elephants during the period from 2013-2017 in the two zoos in Berlin (Berlin Zoologischer Garten and Tierpark Berlin). An EEHV1-specific quantitative polymerase chain reaction (qPCR) assay was used to detect viral DNA in whole blood, urine, and trunk washes. The results are discussed in terms of the significance of routine monitoring and the feasibility of antiviral drug treatment.

All elephants in the study were Asian elephants that were born either in the wild or in captivity (Table 1). Different samples were collected from the elephants throughout the study, as indicated in the table. Not all elephants in the Tierpark were included for the whole period of the study due to the movement of animals between zoos. The young elephant (AZ_2016), the daughter of PZ_2016 and VZ_2016, was born in August 2012 and is the youngest among the Asian elephants in the Berlin Zoologischer Garten. AZ_2016 has been included in the routine EEHV screening program since she was 8 months old. No apparent EEHV-related clinical signs were observed in the elephants, except for AZ_2016, who showed slight irritability at the time of EEHV-1 detection; however, her general condition remained unaffected.

Urine samples were collected from AZ_2016 from April 2013 until May 2016, when sampling using trunk swabs was initiated. For the other elephants, blood samples were collected during the whole study, except for TZ_2016, PT_2016 and TT_2016 (Table 1). Testing of trunk washes in addition to blood samples was done from May 2016 until the present. All of the elephants in the Berlin Zoologischer Garten were sampled twice a month until June 2016, when we started to sample all of the elephants twice a week and AZ_2016 daily. All of the Berlin Tierpark elephants were sampled twice a month.

All samples were collected as part of the routine health screening program and sent immediately for laboratory testing. DNA was extracted using a Stratec DNA extraction kit (Stratec biomedical, Germany) according to the manufacturer's instructions. All DNA samples were kept at -20 °C until analysis by qPCR.

All DNA samples were analyzed for the presence of EEHV-1 sequences by qPCR, using an Applied Biosystems 7500 FAST System (ABI, Germany). Primers and probes (forward primer, ACTGCAAAYGCATTCTTAAAAGAT; reverse primer, AGAATGGGATTRGCTAAGAAGCT; probe, 5-FAM-TCAACGAGGAGATATTAGGCACCA CCAACA-BHQ1-3) were synthesized to target the EEHV-1 terminase gene based on primers and probe sequences described earlier [7]. qPCR protocols for the other EEHV species members (EEHV-2, EEHV-3, EEHV-4, EEHV-5, and EEHV-6) were performed as described earlier [8].

DNA obtained from AZ_2016, PZ_2016 and PT_2016 was subjected to PCR amplification using primers specific for the EEHV-1 terminase gene (FW primer, ATATCA CTTAATTTGAATAT; RV primer, TGATAAAACTGC AAGATACA) to yield a 511-bp pCR product. To amplify the EEHV-1-U51 (vGPCR) gene, we used specific primers

Table 1 Asian elephants included in the study

Name	Zoo	Age (years)	Sex	Sample	Duration of the study	Country of origin
TZ_2016	Berlin Zoo	53	Female	Urine and trunk wash	2013-2017	India
DZ_2016	Berlin Zoo	46	Female	Blood and trunk wash	2013-2017	Vietnam
CZ_2016	Berlin Zoo	43	Female	Blood and trunk wash	2013-2017	India
IZ_2016	Berlin Zoo	43	Female	Blood and trunk wash	2013-2017	India
PZ_2016	Berlin Zoo	29	Female	Blood and trunk wash	2013-2017	Thailand
VZ_2016	Berlin Zoo	23	Male	Blood and trunk wash	2013-2017	Israel
AZ_2016	Berlin Zoo	4	Female	Urine and trunk wash	2013-2017	Germany
CT_2016	Tierpark Berlin	22	Female	Blood	2013-2015	Indonesia
LT_2016	Tierpark Berlin	44	Female	Blood	2013-2016	India
KT_2016	Tierpark Berlin	34	Female	Blood and trunk wash	2013-2017	Myanmar
PT_2016	Tierpark Berlin	4	Female	Trunk wash	2016-2017	Germany
TT_2016	Tierpark Berlin	8	Female	Trunk wash	2016-2017	Germany

kindly provided by Dr. Gary Hayward, Johns Hopkins University, (R1 [LGH7506], GATTGTGAACGCTGTATGTC; R3 [LGH7471], CGGTTACACCGTACCGTGGCTTGC; L1 [LGH4963B], GACTTTCTTCGTGTAGCCCTCGTC TT). In the first-round PCR, we used R1 and L1 to yield a 910-bp product, and in the second-round PCR, we used R3 and L1 to yield a 550-bp product. The amplified products from the terminase (511 bp) and vGPCR (550 bp) regions, were purified and sequenced by the Sanger method (LGC Genomics).

Phylogenetic analysis was based on nucleotide sequences of the terminase and vGPCR genes. Reference sequences for the same regions of EEHV were obtained from GenBank and aligned using ClustalW implemented in Bioedit software [18]. Phylogenetic analysis of the alignments was performed by the maximum-likelihood (ML) method using the MEGA 7.0.26 software [21]. Branching was supported by bootstrapping with 1000 sets of data.

The young elephant AZ_2016 was treated with ganciclovir (GCV) 5 mg/kg [2, 16] by oral administration twice daily for 3 days (Source: Houston Zoo Asian Elephant EEHV Treatment Protocol, April 2015). GCV was injected into bread pieces, mixed with smashed beets, and given by hand to ensure complete uptake. No supportive therapy was given to the animal, since no clinical signs were evident.

DNA extracted from the samples was subjected to EEHV-1 qPCR assay. From February 2013 to April 2016, all elephants tested negative for EEHV-1. The first positive sample dates to May 2016, when a trunk swab from the young elephant AZ_2016 was found to contain a low number of virus copies, as evidenced by a high threshold

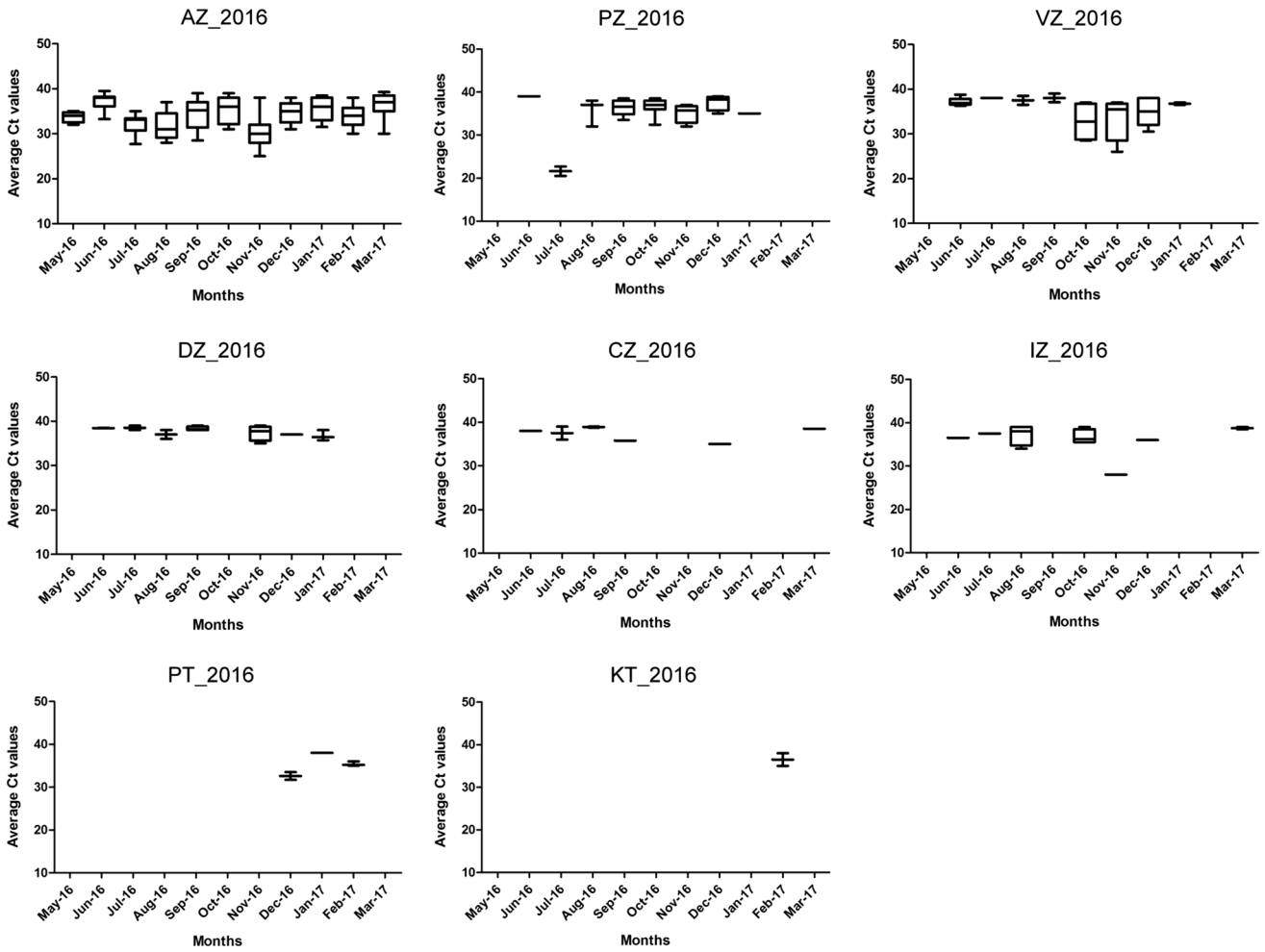

Fig. 1 Elephant endotheliotropic herpesvirus 1 from trunk washes detected by qPCR. Average Ct values obtained from six elephants from the Berlin Zoologischer Garten and two from Tierpark Berlin are shown. The central line in the box plot indicates the median of the data, while the edges of the box indicate the 25th and 75th percentiles. Extending from the box are whiskers. The top whisker expands to the 95th percentile and the bottom whisker to the 5th percentile. All samples were tested in duplicate: Ct value > 40 in both replicates: sample is negative. Ct value ≤ 37 in both replicates: sample is positive. Ct value 38-40 in both replicates: questionable. DNA extraction and qPCR were repeated again, and the sample was considered positive if the Ct value was < 40

cycle (Ct) value (Fig. 1). At this point, all elephants were also tested for other EEHVs (EEHV-2 to 6), but all of those assays were negative (data not shown). Almost all of the elephants (six out of seven) in the Berlin Zoologischer Garten and two out of five in the Tierpark Berlin have had positive Ct values for EEHV-1 (Fig. 1). Interestingly, AZ_2016 had been continuously shedding the virus from trunk secretions over a period of almost one year (Fig. 1), but without showing any clinical symptoms. Other elephants shed the virus in trunk secretions sporadically and for a short period of time (Fig. 1). It is noteworthy that EEHV-1 was detected in trunk samples, but not in the blood.

In order to determine the genetic relatedness of the shed virus to known EEHV-1 sequences, the terminase and vGPCR genes were amplified from DNA obtained from AZ_2016, PZ_2016 and/or PT_2016 and sequenced. The sequences were phylogenetically analyzed using the maximum-likelihood method (Fig. 2). At the nucleotide level, all of the terminase gene sequences (AZ_2016 GenBank accession number MG030498) obtained from the three elephants and the vGPCR sequence obtained from PZ_2016 (GenBank accession number MG030497) belong to an EEHV-1B clade that includes a sequence of the North American strain EEHV-1B (GenBank accession numbers KT705262 and JN633920.1).

Based on qPCR results, the decision was made to start antiviral treatment for AZ_2016. The young elephant was given 9 g of GCV twice daily for three consecutive days during the period from 5-7 June 2016. As shown in Fig. 1, GCV treatment did not affect virus shedding.

We here describe the importance of routine monitoring of elephants in zoos as a useful tool in early detection and tracking of EEHV-1 infection and shedding. EEHV-1 is known to be one of the biggest threats to young Asian elephants, both in captivity and in the wild [9]. Although infection with EEHV-1 is usually fatal, several reports have documented the survival of young Asian elephants after infection [2, 9, 12]. Here, we report on the infection of a young elephant with EEHV-1B that did not show any clear clinical signs and survived the infection. There is no good explanation as to why some young elephants successfully control the infection while others do not. Long and colleagues review and discuss possible reasons [9]. One of the important factors seems to be maternal antibodies, which may give the calf a chance to develop its own antibodies and survive the critical stage following primary infection. Furthermore, the young elephant AZ_2016 was born in the zoo to captive parents, which may support the notion that mixed breeding from parents of diverse origin may give the genetically heterogeneous offspring increased resistance to infection [9]. To support the hypothesis of the role of maternal antibodies, serological assays [19] using samples from both PZ_2016 (the mother) and AZ_2016 (the daughter) can

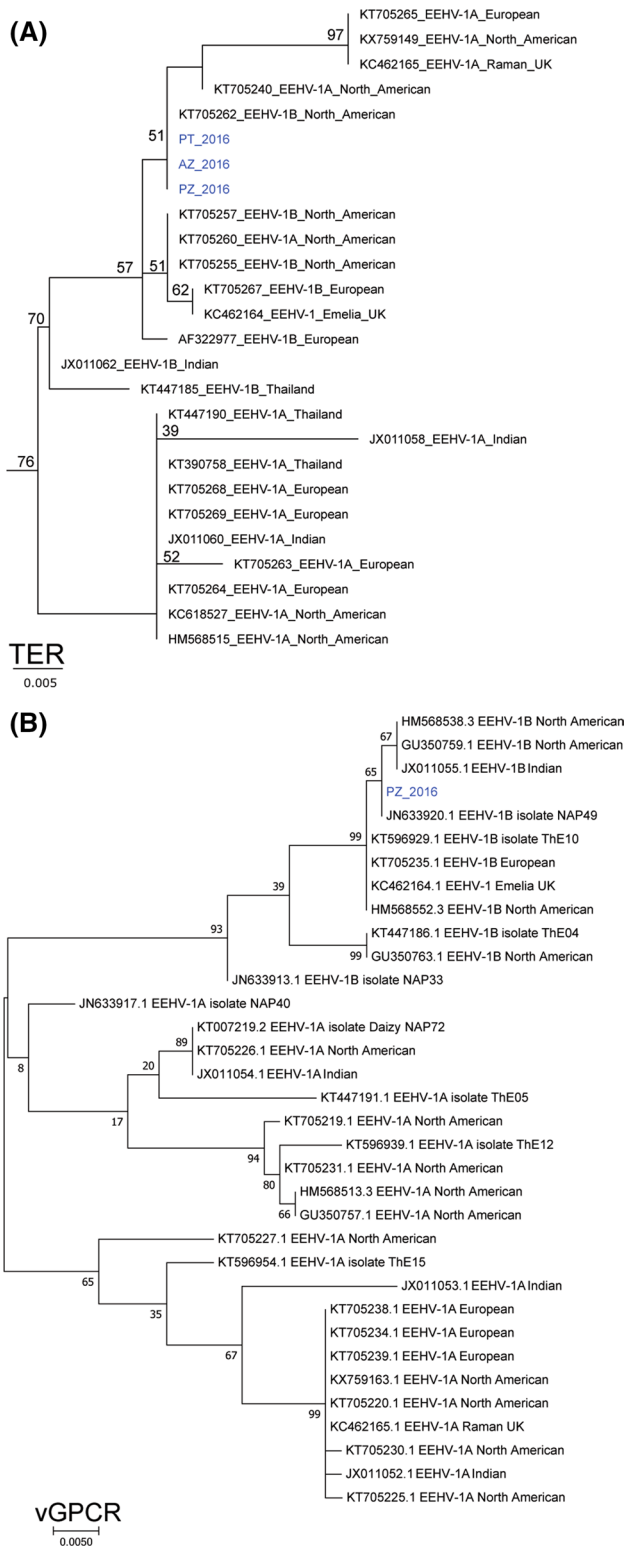

Fig. 2 Phylogenetic tree of EEHV-1 DNA. Maximum-likelihood trees are shown for the terminase (A) and vGPCR (B) genes. AZ_2016, PZ_2016 and PT_2016 are indicated in blue. Sequences obtained from GenBank are represented by their accession numbers. Bootstrap values above 50% are shown. The scale bar indicates nucleotide substitution per site

be used. However, collecting blood samples from AZ_2016 was difficult, as she was not trained for blood collection.

Different samples (blood, urine and trunk washes) were tested throughout the study. However, EEHV-1 was detected only in trunk washes, confirming virus shedding. Our results underscore the importance of trunk washes as the optimal sample types for detection of EEHV in secretions from asymptomatic carriers or subclinically infected elephants [7, 15]. Other samples, including swabs from the conjunctiva, palate and vulva, can also be used [7]. Furthermore, we confirm the feasibility of using the previously established qPCR assay [7] to carry out routine monitoring of elephants in zoos. However, caution should be used, as negative qPCR signals can be obtained if sampling of the animals is done at a time when they are not shedding virus.

A few weeks after the young elephant AZ_2016 was found to be an EEHV-1 shedder, other elephants in the Berlin Zoologischer Garten also started to shed the virus in trunk washes, suggesting that the virus was transmitted from AZ_2016 to the other elephants. Elephants at the Tierpark Berlin were positive for EEHV-1 months after the first detection in the Berlin Zoologischer Garten. However, possible transmission of the virus from Berlin Zoologischer Garten to Tierpark Berlin can be excluded if one takes into consideration the distance between the two locations and the absence of any contact between the two zoos. One possible explanation could be virus reactivation from latent animals. It is worth mentioning that the elephant PT_2016 was introduced to the Tierpark Berlin in October 2016, and she was the first to shed EEHV-1 in trunk secretions in December 2016. We surmise that PT_2016 could have experienced reactivation of latent virus after being exposed to stress factors with subsequent transmission to other elephants (particularly KT_2016) in Tierpark Berlin.

Sequencing of the conserved terminase or the highly variable vGPCR genes [9] demonstrated that these sequences are phylogenetically related to a North American EEHV-1B strain. Our results are in line with those of a previous study in which EEHV-1B was isolated from an 11-year-old male Asian elephant (Kiba) that died in the Berlin Zoologischer Garten in August 1998 of a systemic hemorrhagic disease [4, 5, 12]. Kiba was born in the Houston Zoo in 1987 and moved to Berlin Zoologischer Garten in 1997. It seems likely that the EEHV-1B strain is still circulating in the zoo since the first introduction with Kiba.

The efficacy of using nucleoside, nucleotide, and pyrophosphate analogs as antiviral drugs for EEHV treatment is questionable. FCV, GCV, and acyclovir were used previously to treat "at-risk" elephants, but efficacy was never proven [2]. In the current study, we used GCV in an attempt to reduce viral loads in trunk secretions. As in previous studies, however, we did not notice a significant effect. It is probable that, in our case, the treatment duration (3 days) was not sufficient. However, it is still unclear if GCV treatment helped to avoid a fatal outcome.

Zoological collections represent an artificial system mimicking the natural habitat. Zoos attempt to provide high-quality standards to protect wild animals, especially endangered species that are kept in captivity. However, infectious diseases still a real threat for many captive animals, including elephants. The current and previous findings provide evidence of the prevalence of EEHV among captive Asian elephants. Several zoos have adopted a long-term routine molecular screening program to track the onset of EEHV infection. These programs have helped with early detection and immediate intervention to protect the elephants and with tracking the possible sources and modes of transmission of these viruses.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Sample collection was done during the routinely performed veterinary health checks according to the National Animal Protection Act (Tierschutzgesetz; approval number D-AFF005–EWG).

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