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Research article

Current surveillance practices for shedding of elephant endotheliotropic herpesviruses in breeding and bachelor Asian elephant *Elephas maximus* herds in Europe

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Abstract

Elephant endotheliotropic herpesvirus-haemorrhagic disease (EEHV-HD) is the most common cause of death in juvenile captive Asian elephants *Elephas maximus*. Currently, weekly whole blood screening is recommended for the detection of viraemia, which occurs prior to the development of clinical disease, but there are no recommendations for monitoring viral shedding into the environment. The aims of this study were to evaluate current EEHV shedding surveillance protocols in Asian elephant herds in Europe, as well as to collate and describe existing EEHV shedding data from these herds. Results from a European Association of Zoos and Aquaria Taxon Advisory Group-approved survey revealed that as of January 2021, 42% of breeding institutions had a protocol for screening for EEHV viraemia, while 30% monitored viral shedding. Shedding data were available from 12 institutions, where a total of 2,863 samples had been collected for polymerase chain reaction (PCR) analysis. Overall, 13.9% of all tested samples were positive for EEHV and 48.9% of elephants tested positive for EEHV. EEHV-1 was both the most common genotype detected and the most commonly tested for. Evidence of the presence of EEHV was reported in 12/12 (100%) of breeding herds. Routine monitoring of EEHV shedding is recommended to enable better understanding of the dynamics of EEHV infection and disease.

Introduction

The worldwide Asian elephant *Elephas maximus* population is considered endemically infected with a group of co-evolved elephant endotheliotropic herpesviruses (EEHVs) (Long et al. 2016). While there is relatively little pathology associated with infection in adult elephants (Schaftenaar et al. 2010; Stanton et al. 2010), EEHV is the leading cause of death in juveniles (Perrin et al. 2021a). Clinical disease, which often results in death, is known as EEHV-Haemorrhagic Disease (EEHV-HD) (Howard and Schaftenaar 2019). EEHV-specific polymerase chain reaction (PCR) analysis of whole blood samples can detect viral DNA, which indicates an active viral infection (viraemia). Clinical signs develop as the amount of circulating virus increases, and exponential increases in whole blood viral load have been documented in fatal cases (Seilern-Moy et al. 2016a; Stanton et al. 2013). Because asymptomatic viraemia can be detected prior to clinical disease, the EEHV Advisory Group currently recommends weekly PCR screening of whole blood for the monitoring of juvenile elephants (Howard and Schaftenaar 2019) to enable early initiation of treatment where necessary.

The same PCR assays can be applied to samples of bodily fluids, in order to detect viral shedding into the environment (Hardman et al. 2012; Stanton et al. 2010). EEHV DNA has been detected in samples from the trunk, oral cavity, conjunctiva, vulva and faeces (Common et al. 2022; Hardman et al. 2012;

Jeffrey et al. 2020). Shedding occurs after peak-viraemia in clinical cases (Stanton et al. 2013), as well as intermittently throughout adult life (Ackermann et al. 2017; Stanton et al. 2010).

Asian elephant calves are born with protective maternally-derived EEHV-antibodies (Fuery et al. 2020; Hoornweg et al. 2021). Antibody levels wane during the first years of life, which coincides with the risk period for EEHV-HD death (Fuery et al. 2020; Perrin et al. 2021a). Recent evidence shows that fatal cases do not have detectable levels of EEHV antibodies, while antibodies can be detected in calves that survive (Fuery et al. 2020; Hoornweg et al. 2021, 2022). Overall, this suggests that the temporal relationship between waning maternal antibodies, calf exposure to EEHVs from mothers or herd mates and/or maturation of the calf's own antibody responses may influence the likelihood of developing EEHV-HD. A current, untested hypothesis is that regular calf exposure to EEHVs shed from herd mates may promote the development of calf antibody responses as maternal antibody levels wane. In contrast, a calf that is not exposed to EEHV prior to the disappearance of maternal antibodies may be more likely to develop EEHV-HD, due to a suspected exaggerated innate immune response (Perrin et al. 2021b).

While the amount of EEHV DNA in blood corresponds with the severity of clinical signs, the presence and quantity of shed viral DNA does not seem to have a direct association with disease. Accordingly, there are currently no recommendations for monitoring of EEHV shedding, despite this being the likely source of virus exposure and infection between herd mates. The first aim of this study was to evaluate current EEHV shedding surveillance protocols in captive Asian elephant herds in Europe. Breeding and bachelor groups were included, as these include young elephants considered at risk of developing EEHV-HD. The second aim was to collate and describe existing EEHV shedding data from these herds. It was hypothesised that EEHV would be detected in every Asian elephant herd, and that herds with a history of EEHV deaths would have less frequent detection of EEHV shedding than herds with no history of EEHV death.

Materials and methods

EEHV surveillance survey

After approval by the European Association of Zoos and Aquaria (EAZA) elephant Taxon Advisory Group (TAG), a survey consisting of eight questions (Table 1) was sent to 37 Asian elephant breeding facilities and 9 facilities housing bachelor groups in Europe, between April 2020 and January 2021.

EEHV shedding data

For institutions which indicated that EEHV shedding had been monitored, either routinely or intermittently, and that were willing to contribute data to this project, complete results were requested. Data included elephant identification, sex and age, as well as the type of sample collected (e.g. urine, trunk, ocular) and the method of sampling employed (e.g. swab, wash). PCR results were reported as positive or negative. For quantitative PCR methods, where cycle threshold (Ct) values were available, any value less than 40 was considered positive. Herds were classified as affected or unaffected by EEHV-HD, depending on whether there was a history of EEHV-HD in the herd. Affected herds were further classified as having experienced EEHV-HD deaths, or EEHV-HD but no deaths.

Results

EEHV surveillance

From the 37 breeding facilities approached, 31 (84%) returned the survey, of which 30 were completely answered and one was incompletely answered. Of the 31 participating breeding institutions, 13 (42%) indicated that they had a protocol for EEHV viraemia surveillance while 18 (58%) indicated that they did not. Eleven (35%) breeding institutions indicated that there was an EEHV shedding surveillance protocol in place, and seventeen (55%) reported diagnostic shedding samples had been collected at some point, even if there was no protocol. Of the 14 institutions that reported sample type and/or method, 13 (93%) sampled from the trunk (5/13 trunk wash and 5/13 trunk swab, 3/13 did not report the method), 3 (21%) used conjunctival swabs and 1 (7%) used oral swabs to collect saliva. Two institutions reported sampling from multiple anatomic sites (both trunk and conjunctiva). Of the 11 institutions with a shedding surveillance protocol, 5 (45%) sampled weekly, 1 (9%) sampled every 14 days, 3 (27%) sampled monthly and 1 (9%) sampled twice per year. An additional six institutions monitored shedding intermittently. It should be noted that the presence of a protocol to screen for EEHV viraemia or shedding did not guarantee that samples were collected and/or analysed at the prescribed intervals stated in the protocol. Keepers most commonly collected diagnostic samples (11/17, 65%), followed by veterinary staff (3/17, 18%) or both keepers and veterinary staff (3/17, 18%). PCR analysis was performed either at an external laboratory (15/19, 79%) or using in-house facilities (4/19, 21%).

From the nine approached holders of bachelor groups, three (33%) responded with a completely answered survey. None of these facilities had a protocol for monitoring EEHV shedding, and only one had a protocol for viraemia surveillance, using in-house PCR facilities.

EEHV shedding data from breeding herds

One or more PCR results from samples collected to screen for EEHV shedding wwere available from 12 (32%) of the 37 breeding institutions, and from none of the bachelor herds. In total 2,863 results were available for analysis, from 78 elephants (25 males and 53 females, 52 captive-born and 26 wild-born). Samples were collected between August 2009 and July 2021. The elephant age for samples ranged from 1 month to 55 years (median 225 months, interquartile range 55–456 months). The number of samples per elephant ranged from 1 to 273 (median 9 samples, interquartile range 3–38).

Samples were collected from the trunk (n=2,405, 84.0%), oral cavity (n=246, 8.6%), conjunctiva (n=79, 2.8%) and vulva (n=8, 0.3%). Urine samples were also tested (n=96, 3.4%) and the sample origin was not reported in 29 cases (1.0%). Samples other than urine were submitted as swabs (n=2,682, 93.7%), or trunk washes (n=71, 2.5%).

Of the 78 elephants tested, 38 (48.7%) tested positive for EEHV shedding at least once. Overall, 397/2,863 (13.9%) samples were positive for EEHV, with 391/2,863 (13.7%) samples positive for EEHV-1, 6/995 (0.6%) positive for EEHV-3/4 and 0/59 (0%) samples positive for EEHV-5. Every sample was tested for EEHV-1, but not necessarily for EEHV-3/4 and/or -5. For male elephants, 89/614 (14.5%; n=86 EEHV-1, n=3 EEHV-3/4) samples were EEHV positive while 308/2,249 (13.7%; n=305 EEHV-1, n=3 EEHV-3/4) samples from females were EEHV positive. Ten of the twelve (83.3%) institutions which had screened for EEHV shedding had at least one elephant test positive for EEHV shedding. The remaining two institutions had the lowest number of shedding results available, n=2 and n=9, respectively. Both institutions had a history of detecting EEHV in blood or tissue samples (Ludwig et al. 2014; Perrin et al. 2015; Seilern-Moy et al. 2016b). The shedding data from the current study and previously documented cases of EEHV-HD indicate that 100% of the 12 breeding herds represented in this study had evidence of EEHVs circulating in their herds. Three institutions had no history of clinical EEHV-HD cases (27/151

Figure 1. Elephant endotheliotropic herpesvirus surveillance protocols survey sent to institutions holding breeding and bachelor Asian elephant *Elephas maximus* herds in Europe.

Name of facility		Name of person in charge	
1. EEHV monitoring			
A) Is there a monitoring protocol in place for EEHV shedding (samples other than blood)?			
Yes	No		
B) Which kind of sample are you analysing regarding EEHV shedding?			
Trunk wash	Conjunctival swab	Genital swab	Others, namely:
C) In which interval are you sampling the elephants under your care?			
Weekly	Monthly	Twice a year	Different interval, namely:
D) Who is taking samples at your facility?			
Elephant keepers	Veterinarians	Other person, namely:	
E) Where are your samples analysed?			
In-house lab at the zoo	External laboratory	Location:	
F) Is there a monitoring protocol in place for EEHV viremia (whole blood) at your facility?			
Yes	No		
2. Data records on EEHV shedding status			
A) Are there data (including intermittent single test results) on the shedding status of your elephants available in your records?			
Yes	No		
B) Are you willing to share these data (confidentially and anonymously) and contribute them to the current research?			
Yes	No		

samples positive for EEHV, 17.9%), eight had a history of EEHV-HD deaths (369/2,642, 14.0%) and one had a history of a clinical EEHV-HD case but no deaths (1/70, 1.4%). Statistical evaluation was not performed as institutions with no history of EEHV-HD were under-represented in the dataset and sampling protocols had not been standardised.

One or more shedding test result (n=757) was available from 23 elephants between birth and 8 years of age, which is the risk period for EEHV-HD in Europe (Perrin et al. 2021a). Twelve elephants (52%) were positive for shedding prior to 8 years of age. Age of first detection ranged from 46 days to 55 months (median 16 months, interquartile range 8–34). Four out of seventy-eight elephants (5%) in the sample population died of EEHV-HD. One of these elephants tested positive for EEHV-1 shedding once, five days prior to death caused by EEHV-1A. In total there were 74 samples from the four elephants that died of EEHV-HD. Of these, 1.3% (1/74) tested positive for EEHV shedding compared with 396/2,789 (14.2%) samples from elephants that did not die of EEHV-HD.

EEHV-1 positive samples were distributed as follows: 12/71 (16.9%) trunk washes, 357/2,320 (15.4%) trunk swabs or 1/14 (7.1%) trunk samples obtained 'from the substrate' (trunk secretions or wash fluids collected from the ground) , 2/246 saliva swabs, 2/29 unknown samples and 0/96 urine samples. Additionally, 3/8 (37.5%) vulval swabs and 14/79 (17.7%) conjunctival swabs were positive for EEHV-1. The three positive vulval results and six of the positive conjunctival results have previously been reported (Hardman et al. 2012). This previous study reported that only 3/63 vulval and 6/77 conjunctival swabs were positive for EEHV-1; however, only data from positive results were provided for the current study. The authors of the current study confirmed that the negative results from Hardman et al. (2012) should be included in the present study in order to avoid (positive result) reporting bias. Therefore, the corrected prevalence of EEHV in vulval and conjunctival samples, including data from both studies, is 3/68 (4.4%) and 14/150 (9.3%), respectively.

All EEHV-3/4 positive samples were trunk samples, and one trunk swab was positive for both EEHV-1 and EEHV-3/4. EEHV-5 was not detected in any sample, Urine samples were only tested for EEHV-1.

Discussion

All of the Asian elephant breeding herds that provided EEHV PCR shedding results in this study have evidence of EEHVs circulating in the herds, including three institutions with no history of clinical EEHV-HD cases. These data strongly support the first hypothesis that EEHV is present in every breeding Asian elephant herd in Europe, which is consistent with previous shedding and antibody surveys (Ackermann et al. 2017; Bennett et al. 2015; Hoornweg et al. 2021). EEHV-1 was the most common genotype detected; however, it was also the only genotype tested for in every sample. EEHV-5 was not detected; however, it was rarely tested for, so these data should be interpreted with care. EEHV-4 and -5 are less commonly associated with EEHV-HD death in Europe, which may explain why there has been less emphasis on screening for these

genotypes (Perrin et al. 2021a,b). PCR analysis cannot differentiate between EEHV-3, an African elephant *Loxodonta africana* EEHV, and EEHV-4, an Asian elephant EEHV (Stanton et al. 2012). Samples from Asian elephants that test positive on an EEHV-3/4 PCR assay are assumed to be EEHV-4 positive, but sequencing of the PCR products is required to confirm this. Sequencing is also required to differentiate between EEHV-1A, the most common cause of EEHV-HD death, and EEHV-1B (Perrin et al. 2021a,b). Sequencing results were not reported in this dataset, and sequencing does not appear to be routinely performed on surveillance samples in Europe.

Awareness of EEHV-HD and its impact on the Asian elephant population has been growing since the disease was first described in 1999 (Richman et al. 1999). The number of zoos monitoring for clinical disease has gradually increased as EEHV PCR testing has become more widely available, with 42% now reporting a protocol for EEHV viraemia surveillance. Currently there are no recommendations for monitoring EEHV shedding, and this was reflected by the lower proportion (30%) of institutions with an EEHV shedding protocol. While there is no current evidence that shedding results should influence clinical decision-making when managing an EEHV-HD case, understanding the epidemiology of EEHV infection is hampered by the lack of knowledge of the dynamics of EEHV shedding, infection and development of disease. Establishment of recommendations for sample type and frequency to monitor EEHV shedding would be helpful. Recent investigations suggest that trunk washes are more sensitive than oral swabbing for the detection of EEHV shedding (Grenus et al. 2020). This is in agreement with the findings of the current study, where conjunctival and vulval swabs were less commonly positive than trunk wash and swab samples. EEHV was not detected in urine samples. Training elephants and staff to collect trunk wash samples is more challenging than trunk swabbing, as reflected in this study where swabs accounted for 96% of the trunk samples submitted for PCR analysis. When screening blood for EEHV viraemia, flocked or foam swabs were preferred over cotton-tipped swabs, Whatman® FTA cards or filter paper (Lopez et al. 2017). However, anticoagulated whole blood samples are more sensitive for EEHV DNA detection than any type of swabbed blood sample, and this may also be the case for trunk wash versus trunk swab sampling. Further investigations are required to identify which sample type and method is most sensitive for detecting EEHV shedding.

Survey response from bachelor herds was poor, and no useful conclusions could be derived from the limited data received. While the risk period for EEHV-HD is up until 8 years of age, most elephants that die from EEHV-HD in Europe will do so prior to 4 years (Perrin et al. 2021a). Elephants are rarely moved from breeding herds prior to 4 years of age. While individuals in bachelor herds may potentially have a lower risk of developing EEHV-HD than younger calves in breeding herds, data on the dynamics of EEHV shedding and infection can provide important information on EEHV epidemiology, eventually informing mitigation strategies and transfer recommendations on a population level.

Previous studies of EEHV shedding have typically been longitudinal studies of small herds from a single institution (Bennett et al. 2015; Hardman et al. 2012; Stanton et al. 2010; Titus et al. 2022), or cross-sectional studies with single results from larger groups of elephants (Sripiboon et al. 2020). Multi-institutional reporting of all available EEHV shedding results in the current study has provided an improved sample size of 2,863 samples. However, there are limitations associated with the retrospective nature of the study. Sampling and PCR analysis protocols were not standardised and quality control methods for each lab were not assessed. There is currently no reference laboratory for EEHV PCR analysis, and multiple conventional and quantitative PCR methods are reported in the peer-reviewed literature (Hardman et al. 2012; Pursell et al. 2016; Richman et al. 1999; Sariya et al. 2012; Stanton et al. 2010, 2012). The results that were provided for analysis may have been subject to selective reporting, and results from institutions with no history of EEHV-HD were under-represented. Prospective studies following standardised protocols to monitor EEHV shedding in EEHV affected and unaffected herds are required to test the hypothesis that regular exposure to EEHV from herd mates allows calf immunity to develop, while avoiding the development of clinical EEHV-HD. Recent antibody studies have shown that EEHV-HD fatalities have undetectable EEHV antibodies (Fuery et al. 2020; Hoornweg et al. 2021). Unfortunately, these antibody assays are not commercially available. It would be interesting to know if PCR detection of EEHV shedding in a herd, can be used as a positive prognostic indicator for reduced susceptibility for the development of EEHV-HD.

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