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NONINVASIVE SAMPLING FOR DETECTION OF ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS AND GENOMIC DNA IN ASIAN (*ELEPHAS MAXIMUS*) AND AFRICAN (*LOXODONTA AFRICANA*) ELEPHANTS

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Abstract

Elephant endotheliotropic herpesvirus (EEHV) hemorrhagic disease (EEHV-HD) threatens Asian elephant (*Elephas maximus*) population sustainability in North America. Clusters of cases have also been reported in African elephants (*Loxodonta africana*). Risk to range country elephant populations is unknown. Currently, EEHV detection depends upon sampling elephants trained for invasive blood and trunk wash collection. To evaluate noninvasive sample collection options, paired invasively collected (blood, trunk wash and oral swabs), and noninvasively collected (chewed plant and fecal) samples were compared over 6 wk from 9 Asian elephants and 12 African elephants. EEHV shedding was detected simultaneously in a paired trunk wash and fecal sample from one African elephant. Elephant γ herpesvirus-1 shedding was identified in six

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chewed plant samples collected from four Asian elephants. Noninvasively collected samples can be used to detect elephant herpesvirus shedding. Longer sampling periods are needed to evaluate the clinical usefulness of noninvasive sampling for EEHV detection.

Keywords

Elephant endotheliotropic herpesvirus; elephant γ herpesvirus-1; *Elephas maximus* ; feces; *Loxodonta africana* ; noninvasive

BRIEF COMMUNICATION

Elephant endotheliotropic herpesvirus (EEHV) can cause an acute fatal hemorrhagic disease (EEHV-HD) in young Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants.[5] In North America, EEHV-HD is the leading cause of death in Asian elephants born since 1980, with 58% of calf deaths caused by EEHV.[5] This makes EEHV-HD a major threat to North American Asian elephant population sustainability. Infection and fatality rates are less well documented in wild Asian elephants; however, clinical cases consistent with EEHV-HD suggest potentially high prevalence.[8,13] Comparatively, few EEHV-HD cases have been reported in African elephants: there are no recorded cases of EEHV-HD related deaths in African elephant range countries, but fatal cases have been reported in captive African elephants in North America.[1,5]

Seven species of EEHV are recognized: EEHV 1, 4, and 5 are endemic in Asian elephants, and EEHV 2, 3, 6, and 7 in African elephants.[5] EEHV is asymptomatically shed intermittently by infected elephants, allowing for epidemiologic study of EEHVs in herds in the absence of clinical cases. Viral shedding detection requires collecting nasal secretions via trunk wash, or saliva and mucosal cells via oral swabs; EEHV viremia detection requires whole blood.[9,11] These techniques require extensive training of elephants, and samples are not easily obtained from untrained or wild elephants.

Noninvasive sampling methods that avoid animal handling, such as collection of feces and discarded chewed plants, have been used successfully in primates, rodents, and shrews for detecting herpesviruses.[6,7,14] The objectives of this study were to develop and evaluate noninvasive sampling protocols for detecting herpesvirus DNA shedding in captive Asian and African elephants using discarded chewed plants and feces, and for the collection of elephant DNA for genetic studies.

Samples were collected from 9 Asian elephants at the Houston Zoo once-weekly for 5 wk (April to May 2018), and from 12 African elephants at San Diego Zoo Safari Park once-weekly for 6 wk (May to July 2018). All the Asian elephants were known to be positive for at least one EEHV species, previously confirmed by quantitative real-time polymerase chain reaction (qPCR) using blood; none of the African elephants had been previously tested for EEHV (Molter and Howard, pers comm.).[5,9,10] The Asian elephants (n = 5 female, n = 4 male) ranged from 1 to 48 yr old, and the African elephants (n = 6 female, n = 6 male) from 4 to 28 yr old. Due to differences in herd management at each institution, sampling was not

uniform: whole blood, trunk wash, and chewed hay and browse were collected from each Asian elephant, and oral swab, trunk wash, and feces from each African elephant.

Two milliliters of whole blood were collected from the auricular vein into EDTA-coated tubes. Saliva and oral cells were collected by rubbing Dacron™ swabs along the hard palate for 3 to 5 sec and then placing them into RNAprotect™ (Qiagen Inc, Valencia, CA 91354, USA). Nasal secretions were collected by instilling 50 ml sterile saline into the nares, elevating the trunk for 30 sec, and then allowing the elephant to blow the saline into a Ziploc™ bag. Trunk wash contents were centrifuged, supernatant was removed, and the pellet was stored in cryovials.[11] A maximum of 4.5 g of the most-chewed portions (characterized by tooth impressions and/or a wet appearance) of grass hay and browse (bamboo, oak, mulberry, and elm) were collected from the elephants' mouths by trained keepers and stored in 3 ml of viral transport media. Given the way that elephants chew their food, it was challenging to record which portion of plants were chewed for what length of time, and therefore, chewing times were not evaluated. Three-gram fecal samples were collected from the center and edges of fecal boluses and stored frozen. All samples were stored at −80° Celsius until analysis.

DNA was extracted from blood using QIAamp DNA blood mini kits (Qiagen Inc), from trunk washes, oral swabs, and chewed plants using DNeasy™ blood and tissue kits (Qiagen Inc) using modified extraction protocols, and from feces using GeneMATRIX Stool DNA Purification Kits (EURx Ltd, ul. Przyrodnikow 3, 80-297 Gdansk, Poland).[3,6,7] qPCR was performed on all samples targeting the EEHV1 MDBP gene, EEHV3/4 TER gene, and EEHV5 POL gene in Asian elephant samples, and the EEHV2 POL gene, EEHV3/4 TER gene, and EEHV6 POL gene in African elephant samples.[9,11] EEHV3 and EEHV4 have homologous terminase genes and therefore a single qPCR assay was used.[9] Parallel qPCR reactions were performed for elephant tumor necrosis factor α (TNFα) to determine quality of elephant host DNA present and as a control for qPCR conditions.[10] Samples were considered positive if >10 viral genome equivalents/reaction were detected or if the average cycle threshold was <40.[9]

Using an exogenous internal positive control assay, all chewed plant samples were found to contain PCR inhibitors that prevented DNA amplification via qPCR.[4] As a result, conventional PCR assays (typically less sensitive to PCR inhibitors) designed to detect the herpesviral DNA polymerase (DPOL) gene from α, β and γ herpesvirus subfamilies and mammalian ferritin (to determine quality of elephant host DNA and as a control for PCR conditions) were used to test Asian elephant trunk wash and chewed plant samples and African elephant fecal samples.[2,12] Samples with amplicons of the correct size were sequenced using Sanger sequencing to determine viral species. Sensitivity of elephant DNA detection was calculated for noninvasive sample types considering all samples to contain elephant DNA as the gold standard. Associations between chewed plant type and elephant DNA detection were evaluated using odds ratios (OR).

Among Asian elephant samples, 4.4% (n = 2/45) of trunk washes were EEHV1 positive; 11.4% of hay (n = 5/44) and 2.2% of browse samples (n = 1/45) were positive for *Elephas maximus* γ herpesvirus-1 (EGHV1; GenBank Accession number MT025986; Table 1). All

Asian elephant chewed plant and blood samples were negative for EEHV1, 3/4, and 5. All Asian elephant samples positive for an elephant herpes virus species were positive for either TNFα or mammalian ferritin, with the exception of one of the five chewed hay samples and the chewed browse sample, which were verified by Sanger sequencing.

Among African elephant samples, 7.2% of trunk washes (n = 5/69) and 1.4% of feces (n = 1/70) were EEHV2 positive (GenBank Accession number MT025985), 13% (n = 9/69) of trunk washes and 2.9% (n = 2/70) of oral swab samples were EEHV3/4 positive, and 4.3% of trunk washes (n = 3/69) were EEHV6 positive (Table 2). The EEHV2 fecal sample corresponded with an EEHV6-positive trunk wash from the same elephant on the same sampling date. This EEHV2-positive fecal sample was positive for both TNFα and mammalian ferritin and was verified via Sanger sequencing. Both oral swabs that tested positive for EEHV3/4 corresponded with positive EEHV3/4 trunk washes from the same elephants on the same sampling dates.

Mammalian ferritin was detected in n = 65/70 African elephant fecal samples (sensitivity = 94%), n = 25/45 Asian elephant chewed hay samples (sensitivity = 53%), and n = 15/45 Asian elephant chewed browse samples (sensitivity = 33%). Chewed bamboo samples were associated with a lower likelihood of detecting ferritin compared with other browse types (hay, oak, elm, mulberry; OR = 0.06, $P < 0.01$).

This study demonstrates that EEHV is shed and detectable in African elephant feces. Further investigation of EEHV shedding in feces utilizing larger sample sizes of both Asian and African elephants would help determine whether fecal samples can reliably be used as a noninvasive method for EEHV clinical detection. This would also help to elucidate whether the discrepancy in EEHV species detected between corresponding fecal and trunk wash samples in multiple elephants is suggestive of variable shedding of EEHV in the gastrointestinal tract versus oral and nasal cavities, and whether shedding mechanisms may be tissue specific. This study also demonstrates that elephant herpesviruses (EGHV-1) are detectable in saliva collected from chewed plants. While EGHV-1 is endemic in elephants, previously isolated from genital and blood samples, it has not been known to cause clinical illness or fatalities.[5] This study design, in which different sample types were collected from the different elephant species, may allow for broader field applications, given the convenience of collecting fecal samples from wild African elephants and discarded chewed plants from human-monitored working Asian elephants.

DNA is essential for genetic studies in captive and free-ranging wildlife and is increasingly being used to detect genetic disease markers. Feces were found to be a reliable source of elephant DNA; chewed plants, particularly bamboo, were a less reliable source. In situations where feces are not available, such as during short periods of wild herd observation, discarded chewed plants may be collected for DNA detection, though collecting multiple samples is suggested given the intermittent detection of host DNA.

In conclusion, detection of EEHV2 in an African elephant fecal sample suggests that this method could be used for detecting EEHV in elephants. Additionally, although EEHV shedding was not detected in chewed plant samples collected from elephants known to be

shedding EEHV at collection time, EGHV1 shedding was detected in saliva collected from chewed plants, suggesting that this could be a potential method for detecting orally shed elephant DNA viruses, pending further study.

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Table 1.

Elephant endotheliotropic herpesvirus (EEHV), elephant γ herpesvirus (EGHV), mammalian ferritin, and tumor necrosis factor α (TNFα) results from quantitative real-time polymerase chain reaction (qPCR) and conventional polymerase chain reaction (PCR) on Asian elephant (*Elephas maximus*) trunk wash and chewed plant samples. ND, not done.

	qPCR			Conventional PCR		
Sample type	**No. positive**	**No. tested**	**Positive (%)**	**No. positive**	**No. tested**	**Positive (%)**
Trunk wash						
EEHV1	2[a]	45	4.4	0	45	0
EEHV3/4	0	45	0	0	45	0
EEHV5	0	45	0	0	45	0
Mammalian ferritin	ND	ND	ND	36	45	80
TNFα	27	45	60	ND	ND	ND
Chewed hay saliva						
EEHV1	0	44	0	0	44	0
EEHV3/4	0	44	0	0	44	0
EEHV5	0	44	0	0	44	0
EGHV1	ND	ND	ND	5[b,c]	44	11.4
Mammalian ferritin	ND	ND	ND	25	44	56.8
TNFα	3	45	6.7	ND	ND	ND
Chewed browse saliva						
EEHV1	0	45	0	0	45	0
EEHV3/4	0	45	0	0	45	0
EEHV5	0	45	0	0	45	0
EGHV1	ND	ND	ND	1[c,d,e]	45	2.2
Mammalian ferritin	ND	ND	ND	15[f]	45	33.3
TNFα	0	45	0	ND	ND	ND

[a]The two Asian elephant EEHV1 positive trunk wash samples were shed by two different elephants on the same day. One of those elephants was also shedding EGHV1, detected in chewed hay.

[b]Four of the Asian elephant EGHV1-positive chewed hay samples were shed by one elephant on four sampling dates, and the fifth was shed by a second elephant on one of those same dates.

[c]All Asian elephant samples positive for an elephant herpesvirus species were positive for either TNFα or mammalian ferritin, with the exception of one of the five chewed hay samples and the chewed browse sample.

[d] The EGHV1-positive chewed browse sample was shed by one elephant the same date as another elephant shed EGHV1 in a chewed hay sample.

[e] Chewed mulberry saliva was positive for EGHV1.

[f] $n = 0/12$ (0%) bamboo; $n = 4/9$ (44.4%) oak; $n = 3/10$ (30%) elm; $n = 8/14$ (57.1%) mulberry.

Table 2.

Elephant endotheliotropic herpesvirus (EEHV), mammalian ferritin, and tumor necrosis factor α (TNFα) results from quantitative real-time polymerase chain reaction (qPCR) and conventional polymerase chain reaction (PCR) on African elephant (*Loxodonta africana*) trunk wash, oral swab, and fecal samples. ND, not done.

	qPCR			Conventional PCR		
Sample type	**No. positive**	**No. tested**	**Positive (%)**	**No. positive**	**No. tested**	**Positive (%)**
Oral swabs						
EEHV 2	0	70	0	ND	ND	ND
EEHV 3/4	2	70	2.9	ND	ND	ND
EEHV 6	0	70	0	ND	ND	ND
Mammalian ferritin	ND	ND	ND	ND	ND	ND
TNFα	70	70	100	ND	ND	ND
Trunk wash[a]						
EEHV 2	5	69	7.2	ND	ND	ND
EEHV 3/4	9	69	13.0	ND	ND	ND
EEHV 6	3	69	4.3	ND	ND	ND
Mammalian ferritin	ND	ND	ND	ND	ND	ND
TNFα	65	69	94.2	ND	ND	ND
Feces						
EEHV 2	0	70	0	1[b]	70	1.4
EEHV 3/4	0	70	0	0	70	0
EEHV 6	0	70	0	0	70	0
Mammalian ferritin	ND	ND	ND	65	70	92.9
TNFα	67	70	95.7	ND	ND	ND

[a]Two of the African elephant trunk wash samples from one individual, and one African elephant trunk wash sample from a different individual were positive for more than one EEHV species on the same date.

[b]The EEHV2-positive fecal sample was positive for both TNFα and mammalian ferritin and was verified via Sanger sequencing.