

# Optimization of non-invasive diagnostic techniques for detection of elephant endotheliotropic herpesvirus (EEHV) in Asian elephants (*Elephas maximus*) and African elephants (*Loxodonta africana*)



**UC DAVIS**  
VETERINARY MEDICINE  
Karen C. Drayer Wildlife Health Center

Alison Jeffrey<sup>1</sup>, Tierra Smiley Evans, DVM, PhD<sup>1</sup>, Christine Molter, DVM, DACZM<sup>2</sup>, Lauren Howard DVM, DACZM<sup>3</sup>, Paul Ling PhD<sup>4</sup>, Tracey Goldstein PhD<sup>1</sup>, Kirsten Gilardi, DVM, DACZM<sup>1</sup>

<sup>1</sup>School of Veterinary Medicine, University of California Davis, <sup>2</sup>Houston Zoo, Incorporated, <sup>3</sup>San Diego Zoo Safari Park,

<sup>4</sup>Molecular Virology and Microbiology, Baylor College of Medicine

## Objectives

### Objective 1:

Develop a non-invasive sample collection protocol for the detection of EEHV viral shedding in Asian (*Elephas maximus*) and African elephants (*Loxodonta Africana*) using discarded chewed plants and feces.

### Objective 2:

Determine the best non-invasive sample type for detection of host DNA in Asian elephants.



Asian elephant herd at the Houston Zoo (left) and African elephant herd at San Diego Zoo Safari Park (right). (Photo credits: Stephanie Adams & SDZSP)

## Background

Elephant endotheliotropic herpesvirus (EEHV) causes an acute, fatal hemorrhagic disease (EEHV-HD) in young Asian elephants in North America and Europe, and in Asian elephant range countries. At least 20% of Asian elephant calves born in North America have been affected by EEHV, with a fatality rate of up to 80%. EEHV has seven species: EEHV 1, 3, 4, and 5 are endemic in Asian elephants, and EEHV 2, 3, 6, and 7 are endemic African elephants (1).

EEHV-HD poses a significant risk for the sustainability of the North American Asian elephant population and an unknown, but potentially significant risk for free ranging elephants and elephants in human care in Asian and African elephant range countries.

EEHV is asymptomatically shed, allowing the opportunity to detect EEHV and study the epidemiology of viruses circulating in elephant herds (5). However, existing methods for EEHV shedding detection require the collection of nasal mucus via trunk wash or saliva via oral swab (4). The techniques, as well as whole blood collection for detection of EEHV viremia, require handling of the elephant and extensive elephant training and preclude their use in wild and many managed herds in Asia and Africa.

Non-invasive sampling methods that do not require animal handling have been established for herpesviruses using discarded chewed plants and feces from primates (2,3). These methods have the potential to be used with elephants and if successful, would facilitate the investigation of the epidemiology of EEHV in elephant range countries.

## Project Significance

EEHV-HD is the single greatest cause of mortality in Asian elephants born in North America. Understanding the epidemiology of this virus is critical as it will allow conservation resources to be allocated towards mitigating environmental and/or husbandry related contributions to the disease, and reduce the impact of EEHV on elephants both free ranging and under human care in Asian and African elephant range countries. Elephants are an integral part of human livelihoods, society, and commerce in parts of Asia, making EEHV significant for both people and elephants throughout the region.

## Methodology

**Study population:** Asian elephants (n=9) at the Houston Zoo (HZ) and African elephants (n=12) at the San Diego Zoo Safari Park (SDZSP) (Table 1).

Institution	Species	Age Range	# Female	# Male
Houston Zoo	<i>Elephas maximus</i>	9 months to 48 years	5	4
San Diego Zoo Safari Park	<i>Loxodonta africana</i>	4 to 28 years	6	6

Table 1. Demographics of study population (n=21).

**Sample collection:** Once weekly from each elephant from April-May 2018 at HZ for 5 weeks and May-July 2018 at SDZSP for 6 weeks.

For invasive sample collection to confirm EEHV: 2 ml of blood was collected from an auricular vein into an EDTA anti-coagulated tube; 2 dacron oral swabs to collect saliva were rubbed along the palate for 3-5 seconds; and trunk washes to collect nasal mucus was obtained by pouring 50 ml saline into nares, the trunk elevated for up to 30 seconds, before allowing the elephant to blow saline into a freezer bag. For non-invasive sample collection: the most chewed portions of discarded hay and browse were sampled using a sterile scalpel blade; and fecal samples were taken from the center and edges of one half of fecal boluses.



Collection of trunk wash, oral swab, whole blood, chewed hay, and feces (left to right). (Photo credits: Stephanie Adams & SDZSP)

**DNA extraction:** DNA was extracted from blood samples using QIAamp DNA blood mini kits, from trunk washes, oral swabs, and chewed plants using Qiagen DNeasy™ blood and tissue kits (3,5), and from feces using QIAamp DNA stool mini kits.

**Quantitative real-time PCR (qPCR):** Performed on all blood, oral swab, trunk wash, fecal, and chewed plant samples. qPCR assays targeted genes for: EEHV 1 MDBP (5), EEHV 3/4 TER, and EEHV 5 POL for Asian elephant samples from HZ; EEHV 2 POL, EEHV 3/4 TER, and EEHV 6 POL for African elephant samples from SDZSP (4); and TNF-alpha for all samples as an internal control. Samples were considered positive with >10 viral genome equivalents/reaction.

**Conventional PCR assays:** Degenerate nested herpesvirus and ferritin assays were performed on all HZ trunk wash, feces, and chewed plant sample types. Suspect herpes positive samples were DNA sequenced for confirmation.

**Statistical methods:** Logistic regression was used to assess the association between non-invasive sample type and detection of mammalian ferritin. Sensitivity was used to assess the number of positive EEHV non-invasive samples as compared to the number of positive EEHV invasive samples.



Sample preparation for qPCR. (Photo credit: Brett Smith)

## Results

### Objective 1:

4.4% of Asian elephant trunk wash samples (n=2/45) tested positive for EEHV 1 (Table 2). All Asian elephant blood, fecal, and chewed plant samples tested negative for EEHV 1, 3/4, and 5, indicating these sample types had a sensitivity of 0 for EEHV detection. 11.4% of Asian elephant chewed hay samples (n=5/44) and 2.2% of Asian elephant chewed browse samples (n=1/45) tested positive for elephant gammaherpesvirus 1 (EGHV1).

13% of African elephant trunk wash samples (n=9/69) and 2.9% (n=2/70) of oral swab samples tested positive for EEHV 3/4, 7.2% of trunk wash samples (n=5/69) tested positive for EEHV 2, and 4.3% of trunk wash samples (n=3/69) tested positive for EEHV 6 (Table 3). Positive oral swabs (n=2) corresponded with positive trunk washes. All African elephant fecal samples were EEHV negative, indicating a sensitivity of 0.

Sample Type	# Positive	# Tested	% Positive
Whole Blood			
EEHV 1	0	32	0
EEHV 3/4	0	32	0
EEHV 5	0	32	0
Trunk Wash			
EEHV 1	2	45	4.4
EEHV 3/4	0	45	0
EEHV 5	0	45	0
Feces			
EEHV 1	0	45	0
EEHV 3/4	0	45	0
EEHV 5	0	45	0
Chewed Hay			
EEHV 1	0	44	0
EEHV 3/4	0	44	0
EEHV 5	0	44	0
EGHV1	5	44	11.4
Chewed Browse			
EEHV 1	0	45	0
EEHV 3/4	0	45	0
EEHV 5	0	45	0
EGHV1	1	45	2.2

Table 2. Detection of EEHV 1, 3/4, and 5 viral shedding in Asian elephant whole blood, trunk washes, and feces via qPCR and detection of EGHV1 in Asian elephant chewed plant samples via conventional PCR.

### Objective 2:

Mammalian ferritin (Table 4) was 5 times as likely to be detected in fecal samples compared to other non-invasive sample types (OR=4.999, p=0.00).

Chewed bamboo samples were associated with a lower likelihood of detecting mammalian ferritin (OR=0.058, p=0.007). Mammalian ferritin was detected from chewed hay, hackberry, mulberry, oak, and elm, however the results were not statistically significant.

Sample Type	# Positive	# Tested	% Positive
Oral Swabs			
EEHV 2	0	70	0
EEHV 3/4	2	70	2.9
EEHV 6	0	70	0
Trunk Wash			
EEHV 2	5	69	7.2
EEHV 3/4	9	69	13.0
EEHV 6	3	69	4.3
Feces			
EEHV 2	0	70	0
EEHV 3/4	0	70	0
EEHV 6	0	70	0

Table 3. Detection of EEHV 2, 3/4, and 6 shedding in African elephant oral swabs, trunk washes, and feces via qPCR.

Sample Type	# Positive	# Tested	% Positive
Feces	36	45	80
Chewed Hay	24	44	54.5
Chewed Hackberry/Mulberry	9	15	60
Chewed Bamboo	1	12	8.3
Chewed Oak	4	9	44.4
Chewed Elm	2	9	22.2

Table 4. Mammalian ferritin DNA detection of fecal and chewed plant samples via conventional PCR.

## Conclusions

### Objective 1:

Based on the results of this study to date, our method of fecal sample collection did not detect EEHV shedding in Asian or African elephants – 18 known positive shedding instances of EEHV in traditional sample types, corresponded with 0 positive fecal samples. During sample processing, chewed plant samples were found to have PCR inhibitors that prevented analysis via qPCR on all chewed hay and browse. Due to this limitation, all sample types were also analyzed for herpes virus shedding detection using conventional PCR techniques. PCR inhibitors in chewed plant samples were not found to interfere with conventional PCR. We demonstrated through the detection of EGHV1 that herpesviruses are detectable using chewed plants from elephants. Due to the low number of elephants shedding EEHV during our study period, further investigation is planned with larger numbers of EEHV shedding elephants to conclude whether or not chewed plants and fecal samples can be suggested as a non-invasive alternative for detecting EEHV shedding.

### Objective 2:

For non-invasive elephant DNA collection, feces are a reliable sample source, as fecal samples in this study were significantly positively associated with mammalian ferritin DNA detection using conventional PCR. In contrast, discarded chewed plants are not as reliable a sample source as feces. Discarded chewed bamboo should be avoided when collecting non-invasive DNA samples, as it was negatively associated with ferritin DNA detection. However, if discarded chewed plants are the desired method of non-invasive sample detection, plants such as hay, oak, hackberry, mulberry, or elm would be recommended over plants such as bamboo.



Semi-captive Asian elephant herds in Myanmar (left and middle) and wild African elephant herd in South Africa (right). (Photo credits: Tierra Smiley-Evans & Alison Jeffrey)

## Acknowledgements

Financial support for Alison Jeffrey was provided by the Students Training in Advanced Research (STAR) Program through a UC Davis School of Veterinary Medicine Endowment Fund. Thank you also to: the One Health Institute laboratory, Brett Smith, Victoria Ontiveros, Alex Trembeau-Bravard, Erin Latimer, Jie Tan, Angela Fuery, Martina Stevens, Daryl Hoffman, Stephanie Adams, the HZ veterinarians, veterinary technicians, hospital keepers, and elephant keepers, and the SDZSP elephant team and clinical laboratory.

## References

- Long, S.Y., E.M. Latimer, and G.S. Hayward. Review of Elephant Endotheliotropic Herpesviruses and Acute Hemorrhagic Disease. ILAR Journal. 2016. 56(3): p. 283-296.
- Selman, T.A., et al. Adenovirus and Herpesvirus Diversity in Free-Ranging Great Apes in the Sangha Region of the Republic of Congo. PLOS ONE. 2015. 10(3): p. e0118543.
- Smiley Evans, T., et al. Detection of viruses using discarded plants from wild mountain gorillas and golden monkeys. American Journal of Primatology. 2016. 78(11): p. 1222-1234.
- Stanton, J.J., et al. Development and validation of quantitative real-time polymerase chain reaction assays to detect elephant endotheliotropic herpesviruses-2, 3, 4, 5, and 6. J Virol Methods. 2012. 186(1-2): p. 73-7.
- Stanton, J.J., et al. Detection of pathogenic elephant endotheliotropic herpesvirus in routine trunk washes from healthy adult Asian elephants (*Elephas maximus*) by use of a real-time quantitative polymerase chain reaction assay. Am J Vet Res. 2010. 71(8): p. 925-33.

