Quiescent Infections of *Elephant Endotheliotropic Herpesviruses* and *Elephant Gammaherpesviruses* Detected in African Elephants in Botswana, Gabon, Kenya, South Africa and USA

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ABSTRACT
In this study, initiated in 2009, quiescent infections of *African Elephant Endotheliotropic Herpesviruses (EEHVs*) *EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A* and *EEHV7B,* that can cause lethal *Elephant Hemorrhagic Disease* (*EHD)* and *African Elephant Gammaherpesviruses (EGHVs) EGHV1B, EGHV2, EGHV3B, EGHV4B* and *EGHV5B,* that are not associated with severe morbidity, were detected in African elephants, *Loxodonta africana* and *Loxodonta cyclotis,* in Africa and USA. We genetically evaluated trunk fibropapillomas (nodules), saliva, blood and tissues collected from 43 wild *Loxodonta africana* (African savannah elephants) in Botswana, Kenya and South Africa; saliva received from 25 wild *Loxodonta cyclotis* (African forest elephant) in Gabon; and saliva collected over a seven year period from 7 wild-born *Loxodonta africana* (6 from Uganda and 1 from Zimbabwe) residing in Six Flags Safari Park in Jackson, NJ, USA, and saliva, blood and tissues collected from an additional 200 *Loxodonta africana* and 100 *Elephas maximus* (Asian elephant) in USA. DNA was extracted and amplified by conventional PCR (cPCR) using three-round nested primer sets targeting many *African Elephant EEHVs* genes loci and primers designed specifically to recognize *African Elephant EGHVs* species. Sanger sequencing yielded unambiguous positive genetic matches to known *African Elephant EEHV2, EEHV3A, EEHV*6, *EEHV7A*, *EGHV1B, EGHV2, EGHV3B, EGHV4B, EGHV5B* and discovered novel species including *EEHV3B*, rare subtypes *EEHV3C to EEHV3H* and *EEHV7B,* and the prototype *African Elephant EGHV1B.* Our extensive library of elephant viral sequences and genomic DNA samples are invaluable assets for development of vaccines against *EHD* and for conserving genomic diversity in dwindling herds in situ and isolated herds in preserves and zoos worldwide.

Key Words: *Loxodonta africana, Loxodonta cyclotis, Elephant Endotheliotropic Herpesviruses Elephant Gammaherpesviruses.*

INTRODUCTION
In 1971, McCully described white lymphoid nodules containing Cowdry Type A intranuclear inclusions considered pathognomonic of herpesvirus disease in the lungs of 74% of 50 *Loxodonta africana* killed during a culling operation in Kruger National Park, South Africa [1]. In 1986, Jacobson described raised proliferative cutaneous lesions (nodules) that contained viral nuclear inclusion bodies morphologically consistent with herpesviruses on 25% of 99 juvenile *Loxodonta africana* imported from Zimbabwe to Florida and Texas in 1982-84 [2]. Douglas-Hamilton had previously noted similar nodule-like warts on wild *Loxodonta africana* in

Manyara, Tanzania in 1975 [3]. In 2009, VR Pearson documented on wild *Loxodonta africana* in Samburu, Kenya, raised fibropapillomas (nodules) similar to those described by Jacobson and Douglas Hamilton [4].

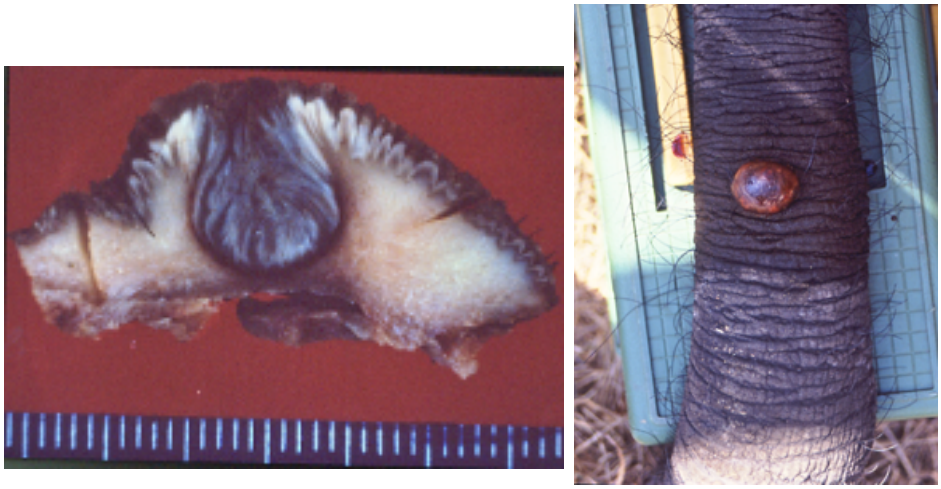

FIGURE 1. Nautilus 99 trunk nodule and cross section on juvenile *Loxodonta africana* imported to America from Zimbabwe in 1982-1984, *photos courtesy Elliott Jacobson, PhD, DVM, University of Florida, College of Veterinary Medicine.*

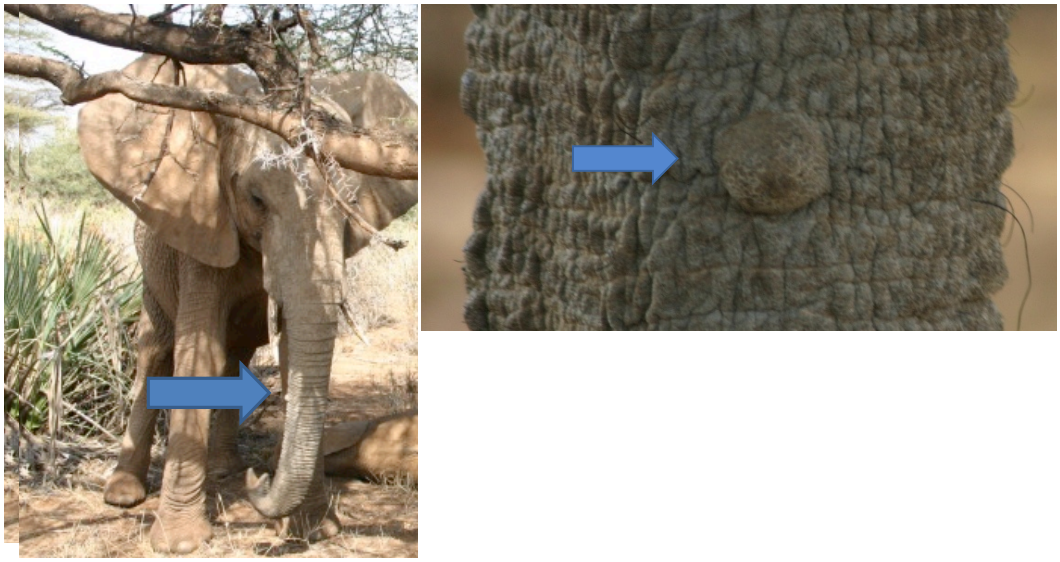

FIGURE 2. VR Pearson photographed nodules on wild *Loxodonta africana* in Samburu National Reserve, Kenya.
FIGURE 3. Close up of nodule. *Copyright VR Pearson, 2009*.

Subsequently, in 2011, with Save The Elephants and Kenya Wildlife Service Veterinary Capture and Services Unit, Kenya, and, in 2013, with Elephants Without Borders, Botswana, nodule biopsies, blood and saliva from 7 wild juvenile and sub-adult *Loxodonta africana* were collected and imported to USA for genetic evaluation. These included: two young calves, designated

KENYA calfGM (in Samburu National Reserve, Kenya) and KENYA MMM (in Maasai Mara National Reserve, Kenya) with two nodules each; one young calf designated KENYA calfMT (Samburu) with one nodule; and two sub-adults designated KENYA HIM (Samburu) and KENYA MMF (Maasai Mara) with one nodule each, (Samburu National Reserve, Kenya, GPS-coordinates: 0°37'5"N, 37°31'48"E and Maasai Mara National Reserve, Kenya, GPS-coordinates: 1°35' 9.00"S, +35°15' 6.00"E; and two juveniles, designated BW1M with 4 trunk nodules and BW2F with one nodule, in Chobe National Park, Kasane, Botswana, GPS-coordinates: 17°47'42.83"S, 25°10'16.067"E. Four mothers (designated KENYA mGM and KENYA mMT (Samburu) KENYA mMMM (Maasai Mara), mBW2F (Chobe) of the youngest calves were also immobilized and saliva and blood collected first to be able to approach the subsequently immobilized calves without dangerous protective interference from their mothers.

Additional samples were collected and exported with the above including: in Kenya, lung biopsies, designated KENYA SAM6 and SAM7, extracted within an hour of death from a well-known sub-adult female *Loxodonta africana* named Enthusiasm who had been killed by ivory poachers in northern Kenya just outside Samburu National Reserve; saliva swabs collected from four *Loxodonta africana,* in Samburu National Reserve, designated KENYA Resilience and KENYA Honesty, who were immobilized for emergency medical treatment of gunshot wounds, and KENYA Mercury and KENYA Taurus, who were immobilized for GPS battery replacement; and in Botswana, saliva swabs collected from an additional 19 *Loxodonta africana*, designated BWABU Sirini, Lerato, Paseka, Kitti, Cathy and BWLA1 to BWLA9, who were accustomed to minimal human contact in tourist camps; and BWCH1 to BWCH5, who were immobilized for GPS collar battery replacement [5-7].

Calf Graca Michele (cGM),
two nodules on top and side of trunk

Calf Mary Todd (cMT)
underside middle third of trunk

Himalaya (HIM,)
topside lower third of trunk

FIGURE 4. 6mm nodule biopsies were collected by VR Pearson in collaboration with Save The Elephants, Kenya and Kenya Wildlife Veterinary and Capture Services. Nodules were positive for *EEHV3A, EEHV3B, EEHV3C, EEHV7A, EEHV7B* and *EGHV1B, EGHV2, EGHV3B.* *Copyright VR Pearson, 2011*

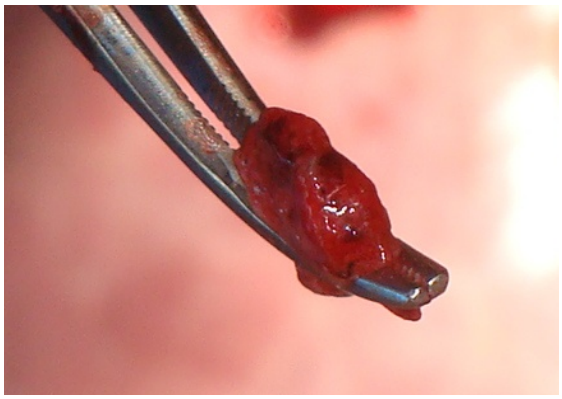

FIGURE 5. Enthusiasm lung was collected and sectioned by VR Pearson during field necropsy.
FIGURE 6. 6 mm lung cubes, designated KENYA SAM6 and SAM7, were immersed in Qiagen

RNAlater. Lung was positive by cPCR for *EEHV2, EEHV3* and *EEHV6. Copyright VR Pearson, 2011.*

FIGURE 7. 6mm nodule biopsies were collected by VR Pearson in collaboration with Elephants Without Borders, Botswana. Wild juvenile male *Loxodonta africana*, designated BW1M. .FIGURE 8. Nodules designated BW1M Nod1 to BW1M Nod4 on underside of trunk. Nodules were positive for *EEHV3A, EEHV3B, EEHV7A, EEHV7B* and *EGHV1B, EGHV2, EGHV3B. Copyright VR Pearson, 2013*

Further, in 2013, with Elephants Alive, Mpumalanga Province, South Africa, biopsies of non-nodular ear warts and saliva were collected from a wild adult bull *Loxodonta africana,* designated RSA Wild Spirit, and saliva only from two adult bulls, designated RSA Proud and RSA Matambu, in Timbavati Reserve, adjacent to Kruger National Park, who had been immobilized for GPS collar battery replacement, GPS-coordinates: 24°20'07S,31°20'38"E. Additionally, trunk nodule biopsies and saliva from five sub-adult South African *Loxodonta africana,* designated RSA BussA Nod, RSA BussB Nod , RSA Buss Sal 3,4,5, that had been collected by South African National Parks Veterinary Wildlife Services at Crocodile Bridge, Kruger National Park, South Africa, 2012, GPS-coordinates: 25°21'30"S, 31°53'32"E, were received and exported to USA along with the samples above that were collected in Botswana and South Africa for this study. And in 2017, saliva samples from 25 African forest elephants, *Loxodonta cyclotis,* designated GA1 to GA25, that had been collected by Agence National des Parcs Nationaux, Gabon, 2017, GPS-coordinates: Minkebe NP 1°40'47"N,12°45'34"E; Mwagna NP 0°36'N,12°42'E; Ivindo NP 0.088°N, 12.63°E; Pongara NP 0°07'N,9°38'E; Loango NP 2°10'S,9°34'E; Moukalaba Doudou NP 2°26'S, 10°25'E, were exported from Gabon to us for genetic evaluation in USA.

From 2012 through 2019, with Six Flags Safari Park, Jackson, NJ, USA, GPS-coordinates: 40.1361^{0}N, 74.4362^{0}W, bi-weekly saliva samples were collected from two of seven wild-born female African savannah elephants from Uganda and Zimbabwe, GPS-coordinates: Chambura 00.70^{0}S, 30.04^{0}E; Murchison 2.2785^{0}N, 31.6855^{0}E; Semliki/Toro 0.4132^{0}N, 34.1054^{0}E, and

Zimbabwe, GPS-coordinates: Sengwe 22.3135^{0}S, 31.2144^{0}E. Periodic saliva samples were collected from the entire herd of seven from 2012-2019 for a total of 170 individual saliva samples. VR Pearson developed the saliva collection protocol because this herd was unaccustomed to diagnostic trunk wash or blood collection protocol commonly used in many USA zoos to detect systemic *EHD* viremia in *Elephas maximus* and more recently *Loxodonta africana* [8,9].

FIGURE 9. USA SFJ Joyce, Six Flags Safari Park, Jackson, NJ, USA, 2012. Puritan 16" saliva swabs were collected from seven *Loxodonta africana* by Jason Holloway, Six Flags Safari Park, and VR Pearson from 2012 to 2019. *Copyright VR Pearson, 2012.*

Samples in Africa were collected under authorization of Kenya Research Permit #NCST/RRI/12/1/MAS/9, Permittee Virginia R. Pearson, and Kenya Wildlife Service Veterinary Capture and Services Department and Elephants Without Borders Botswana Research Permit/ #WT8/36/4XV(41) Researcher Virginia R. Pearson; and Elephants Alive Research Agreement, SAN Parks, South Africa, Researcher Virginia R. Pearson. Samples were exported from Africa and imported into USA under authorization of United States Veterinary Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors #11798, Permittee Virginia R. Pearson; San Diego Zoo Global CITES/ USFWS Import Permit # 13-US727416/9 and #10-US727416/9; International Elephant Foundation CITES/USFWS Import Permit #17US09806C/9; Botswana CITES Export Permit #0202118; Gabon CITES Export Permit #0785; Kenya CITES Export Permit # 008624/ OR82872; and South Africa CITES Export Permit #108757.

Sample collections in USA followed Animal Welfare and Research Committee Protocols and Association of Zoos and Aquariums Elephant Research and Necropsy Protocol. Protocols for elephant immobilization and sample collection were reviewed by San Diego Zoo Global Institutional Animal Care and Use Committee IACUC #11–002.

GENETIC EVALUATION

VR Pearson worked with these samples first in the Enquist Laboratory, Princeton University, Princeton, NJ, USA, and subsequently in the Rall Laboratory, Fox Chase Cancer Center. Neither

lab had previously ever worked with elephant herpesviruses. DNA was extracted and amplified by conventional PCR (cPCR) using three-round nested primer sets targeting many *African Elephant EEHVs* loci including U38(POL), U39(gB), U47(gO), U66(TERex3), U77(HEL), U48.5(TK), U51(vGPCR1, U71/gM, U73(OBP), U77(HEL), U81(UDG), U82(gL), E24(vOX2B1), E4(vGCNT1), E37(ORF-0ex3), E9A(vOGT), E16D(vECTL), E54(vOX2-1), U14, U42(MTA) and U43(PRI) and primers designed specifically to recognize *African Elephant EGHVs* species *EGHV1B, EGHV2, EGHV3B, EGHV4B* and *EGHV5B*.and *Asian Elephant EGHV4A.* Many other primer combinations were used to distinguish unique polymorphisms of subtypes *EEHV3A-EEHV3H,* including chimeric domaines CD-I U39(gB), CD-II U47(gO), U48.5(TK), U48(gH), CD-III U81(UDG), U82(gL) E37(ORF-Oex3) CD IV E9A(vOCT) CD-V E16D(vECTL), among others. Sanger sequencing yielded unambiguous positive genetic matches to *African Elephant EEHV2, EEHV3A, EEHV6, EEHV7A, EGHV1B, EGHV2, EGHV3B, EGHV4B, EGHV5B* and discovered novel species including *EEHV3B*, rare subtypes *EEHV3C-H* and *EEHV7B,* and the prototype *African Elephant EGHV1B.* Many of the primer sets used could also have detected the *Asian Elephant EEHV1A, EEHV1B, EEHV4, EEHV5* if present in the nodule biopsies and saliva but they did not [10-22].

For independent DNA extraction and cPCR and Sanger sequence verification, a subset of nodule biopsies was sent to the Hayward Laboratory, Johns Hopkins University School of Medicine, and yielded identical results. Our evaluation of bi-weekly and sequential saliva samples collected from seven Six Flags Safari Park *Loxodonta africana* and a subsequent study of a nine-month bi-weekly saliva collection from a herd of four *Loxodonta africana* at Maryland Zoo in Baltimore, MD, USA, which included a survivor of *EHD* viremia, also did not detect any of the *Asian Elephant EEHVs* or *EGHVs* [23].

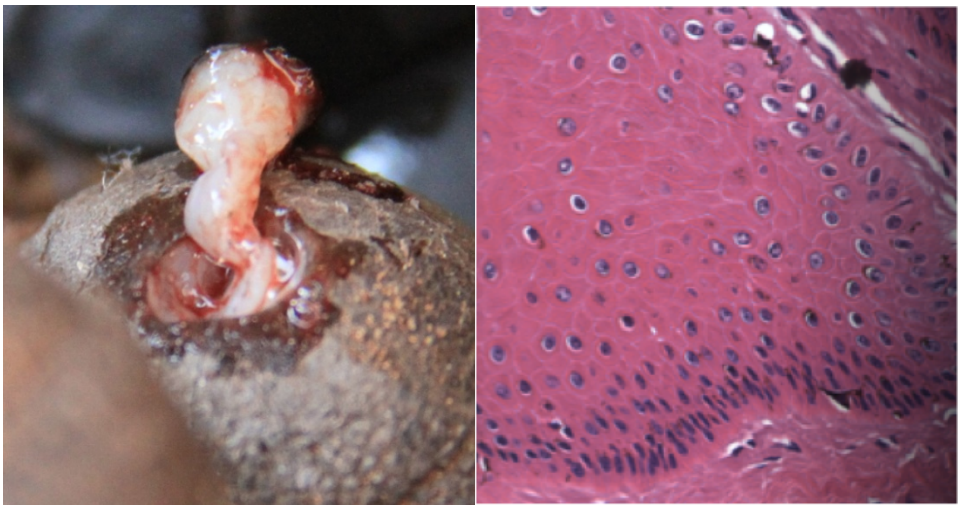

FIGURE 10. Left, nodule BW1M NOD 1. 6mm punch biopsy was collected by VR Pearson, Botswana, 2013, and immersed in Qiagen PAXgene Tissue Container for subsequent FFPE.
FIGURE 11. Right, FFPE block was prepared by SY Long at Hayward Laboratory, Johns Hopkins University School of Medicine, USA, and was cut at 5 microns and stained with hematoxylin and eosin. Histological tissue slide shows multiple keratocyte nuclei that are expanded by a dark basophilic material (intranuclear inclusions indicative of viral infection) distorting the nuclei into an oval to rounded appearance surrounded by a 1-2 um clear halo. mag 45x.

METHODS AND MATERIALS

Sample collection

Wild *Loxodonta africana* and *Loxodonta cyclotis* were immobilized by qualified, experienced field veterinarians using etorphine hydrochloride (M99). Diprenorphine hydrochloride or naltrexone reversed sedation within 90 sec. 5 mm punch biopsies were excised from trunk nodules and immersed in 2.5 ml RNALater Reagent (Qiagen). Incisions were treated with iodine and oxytetracycline. Saliva samples from immobilized, or semi-captive wild or captive elephants in Africa and USA were collected using 16 in polyester swabs (Puritan Medical Corporation) until saturated then immersed in 2 ml RNAprotect Cell Reagent or RNALater (Qiagen Corporation) or collected on GenTegra Ahlstrom GenSaverCards for Blood and Saliva (Gentegra Corporation), all requiring no refrigeration.

DNA Extraction

DNA was extracted from trunk nodule biopsies, saliva, and tissues using Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions with the modification of extended overnight incubation at 56℃, followed by 10 min incubation at 95℃. Archived (FFPE) biopsies, were re-embedded in fresh paraffin after which 8 mm microtome slices per biopsy were incubated in xylene at room temperature for 5 min shaking and rinsed with 100% EtOH a total of five times, prior to DNA extraction.

Polymerase chain reaction

For each 25 µl PCR reaction, we used 2 µl elephant DNA, 12.5 µl GoTaq G2 Hot Start Green Master Mix (Promega), 8 µl sterile nuclease-free H_2O, and 1.25 µl each forward and reverse primers specific for five AelPyV-1 genes. Reactions were run on an Eppendorf MasterCycler or Finnzymes PIKO Thermocycler programmed at 95℃ for 2 min; 45 cycles of 95℃ for 40 sec, 50℃ for 45 sec; 73℃ for 60 sec; final extension 78℃ for 7 min. 25 µl of each PCR reaction was run on 2% agarose electrophoresis gels with ethidium bromide or GelRed or GelGreen nucleic acid stain (Phenix Research Products). Bands of expected size were purified using QIAquick Gel Extraction Kit (Qiagen).

Sequencing

Primer extension sequencing was performed by Genewiz, Inc (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer.

Primers

<u>SET 1 U38 PAN POL</u>

R1 LGH6711 5'-GTATTTGATTTYGCNAGYYTGTAYCC-3'
R2 LGH6712 5'-TGYAAYGCCGTNTAYGGATTYACCGG-3
L1 LGH6710 5'-ACAAACACGCTGTCRGTRTCYCCRTA-3'
rd1 R1/L1 = 530-bp; rd2 R2/L1 = 250-bp.

<u>SET 2A U60 PAN TER</u>

R1 LGH6671B 5'-GTTYGTAGTAAANGCCGGATCTAC-3 '
R2 LGH3025B 5'-GGTACTATATCTTATCATRTC-3'
R3 LGH-R3alt 5'-GGGGTTTGCCAGGAAGCTAGTG-3'
L2 LGH-L2-2A 5'-GTAATCTCSTATGTGTGYGAGGAG,C-3
L1 5' LGH7576B 5'-GCNCACGTRAAGAARACACCG,AC-3'
rd1 R1/L1 = 360-bp, rd2 R2/L1 = 290-bp, rd3 R3/L1 = 210-bp.

<u>SET 4 U71/(gM)</u>

R1 LGH6749 5'-CTATGGGATCCGAACTTTC-3'

R2 LGH6750 5'-CTTTCTAAGGGGGTTTGTTGC-3'
R3 LGH6793 5'-GCCATCGGGATCCGGAAAACGCC-3'
L3 LGH6792 5'-GGGCATCTTCTTCTCCCTCATAGC-3'
L2 LGH6752 5'-CTACATGCCCATGCAGATAGG-3'
rd1 R1/L1 = 750-bp, rd2A R2/L1 and rd2B R1/L2 =730-bp, rd3 R2/L2 = 710-bp,
alt rd 2B R1/L3 = 680-bp and alt rd2C R3/L1 = 700-bp;

SET 5 U51 (vGPCR1) specific for *EEHV2* (not good for *EEHV5*)

R1 LGH7506 5'-GATTGTGAACGCTGTATGTC-3'
R2 LGH7470B 5'-GACAGGTGGTACTGTATGATGTGC-3'
R3 LGH7471 5'-CGGTTACACCGTACCGTGGCTTGC-3'
L3 LGH5201 5'-GCCAGGGTAGATAGAATCAAGGGAA-3'
L2 LGH5200A 5'-CGTGATACGCTTCCAAACATACAGC-3'
L1 LGH4963B 5'-GACTTTCTTCGTGTAGCCCTCGTCTT-3'
rd1 R1/L1 = 910-bp, rd2A R1/L2 = 750-bp, rd3A R1/L3 = 690-bp, and rd2B R2/L1 = 730-bp, rd3B R3/L1 = 550-bp or R2/L2 = 570-bp.

SET 5B U51(vGPCR1) specific for *EEHV1* only (outside pair OK for *EEHV6*)

R1 LGH9323 5'-GTGCTAAACCTTTTCAACGAGACTTC-3'
R2 LGH9351 5'-GCGTTTAACGCMACRCAGTTGGT-3'
L2 LGH9353 5'-CGTAACAGGTTAGCATGAGTATCCTCTTCC-3'
L1 LGH9325 5'-GAGAACGCGTTCTGACTTTCTTCATCA-3'
rd1R1/L1 = 1100-bp; rd2 2A PCR R1/L2 = 990-bp, rd 2B PCR R2/L1 = 1070-bp; rd3AB R2/L2 = 960-bp.

SET 6 U77 PAN HEL

R1 LGH6649 5'-CCAGTCAACGTATAGCTCGTAG-3
R2 LGH6743 5'-GCAAGGTRGAACGTATCGTCG-3
L3 LGH7990 5'-CACCCACCCARTTGTAGGGAAAGTGC-3'
L2 LGH7885 5'-CTGCGTGTAACATGTGTTC-3'
L1 LGH3198 5'-CACAGMGCGTTGTAGAACC-3'
rd1A R1/L1 = 980-bp, rd2A R1/L2 = 950-bp, rd2B R2/L1 = 685-bp, rd3A R1/L3 = 500-bp, rd3B R2/L2 = 540-bp

SET 6B U77(HEL) specific for GC-rich *EEHV3/4/7*

R1-7' LGH7920B 5'-CATGTTSAGGTAGTGCAC**C**GA**G**AGC-3'
R2-7 LGH7921 5'-CTGAACATGTGTTCGGCCAGCAAGG-3'
L2-7 LGH7922 5'-CAGCAGCTAGGAGACGTGGCGAC-3'
L1 LGH3198 5'-CACAGMGCGTTGTAGAACC-3'
rd1 R1-7'/L1 .900-bp, rd2A R1-7'/L2-7 = 500-bp, rd2B R2-7/L1 = 650-bp, rd3AB R2-7/L2-7 = 370-bp.

SET 7A U60 TER specific for *EEHV3/4*

R1 LGH6707 5'-GTGCTGTAGCGGATCATGTC-3'
R2 LGH6727 5'-GCAACACGAGCACGCAAAGTACGTC-3'
L2 LGH6728 5'-CGGATCATGTCGAACTCCGTG-3'
L0 LGH-LO-7 5'-CGTCGAACACGAGCACGCAAAGTACGTC-3'
rd1 1A R1/L1 = 310-bp, rd2A 2A R2/L0-7 = 300-bp, rd2B R1/L2 = 300-bp, rd3A/B R2/L0-7 = 280-bp.

SET 8 U38 POL specific for *EEHV2*

R1 LGH7440 5'-GACTTCGCCAGCTTGTATCC-3'

R2 LGH7437 5'-GTATCATCAAGCTTATAACC-3'
L2 LGH7450 5'-CTCTACATTTACCGTACACTC-3'
L1 LGH6525 5'-CACATCGATACGGAATCTC-3'
rd1 R1/L1 = 510-bp, rd2A R2/L1 = 490-bp, rd2B R1/L2 = 490-bp, rd3AB R2/L2=470bp.

SET 8A U38 POL specific for *EEHV2/5*

R1 LGH8423 5'-CATATACGAATGTGCCTCRGAATAYGAGC-3'
R2 LGH8424 5'-GACATGTATCGYGTGTGYATGGATAAGG-3'
L2 LGH8425 5'-CCCGTGTTAGTGGTGACAGTCGTAAC-3'
L1 LGH8426 5'-CCGCCAACCAGGATGTAAGAAGTTG-3'
rd1 R1/L1 = 890-bp, rd2A R1/L2 = 630-bp, rd2B R2/L1 = 555-bp, rd3AB R2/L2 =450-bp.

SET 9 U38 POL specific for *EEHV3/4*

R1 LGH7400 5'-CAGCATCATCCAGGCCTACAAC-3'
R2 LGH6720 5'-ATCCTGGCGCAGCTGCTGAC-3'
R3 LGH6721 5'-CTCACCTGCAACGCCGTCTA-3'
L1 LGH6719 5'-CGTTGAAGGTGTCGCAGAT-3'
R1' LGH8750 5'-CTGCTACTACTCCACCCTCGTSCTGG-
L1' LGH8751 5'-GCCGTGACGGACTCGGCCACGGCCAG-3'
rd1 R1/L1 = 390-bp, rd2 R2/L1 = 270-bp, rd3 R3/L1 = 15-bp, and alt rd2A R1'/L1 = 360-bp, alt rd2B = R1/L1' = 330-bp, alt rd3A R1'/L1' = 300-bp

SET 12A *EGHV* PAN POL (first step)

R1 LGH6784B 5'-GTGGTKGACTTTGCYAGCCTSTACCC-
R2 LGH6785 5'-CCCMAGYATTATWCAGGCMCA-3'
L1 LGH7489 5'-GTCRGTGTCYCCGTAGAYNAC-3'
rd1 R1/L1-=490 bp, r2 R2/L1 470 bp.

SET12B POL specific for *EGHV1,2,3,4,5* (second step)

All use rd1 R1/L1, rd2 R2/L1, can add rd3 R2/L2 and R2/L3

EGHV1A/B R1-1 5'- CTYTACTGACTCCTGAAGTACTCC-3'
EGHV1A/B R1-2 5'- GYCATCCAGGCCTCAAACCGGGTG-3'
EGHV1A/B L1-2 5'-GTCAAGGCCTGCACCGATGCTGGTG-3'
EGHV1A/B L1-1 5'-CCTAAAGTGGGCTTCGGGATCTCC-3'
EGHV2 R2-1 5'-CCCTCATAACATCAAAGGAGCTAC-3'
EGHV2 R2-2 5'-CACCCAGAACTAAAGGCGGAGGTG-3'
EGHV2 L2-1 5'-CTTTGATAAGGGATAAGGGATTACAG-3'
EGHV3A/B R3-1 5'-CCCAYGAGAAACTACAYATGC-3'
EGHV3A/B R3-2 5'-GCATGGCAATCTAARRCCTGAG-3'
EGHV3A/B L3-1 5'-CTGAATCTGGCATCCGGATCATGTTG-3'
EGHV4 R4-1 5'-CCCTAATTTCCCACGAGAAACTG-3'
EGHV4 R4-2 5'-CATGCATGGTGACCTAAGGCCCG-3'
EGHV4 L4-1 5'-GACCCTAAATCTTGCATTTGGCTC-3'
EGHV4 NEW L4-2 5'-CTGAGGGCGTCTGGGGTAATTGACTC-3'
EGHV4 NEW L4-3 5'-CACAGGGAAGAACACCAGATGCCACACC-3'
EGHV5A/B R5-1 5'-CCCTCATTCCACACAATGAGCTTCATTTGC-3'
EGHV5A/B R5-2 5'-CATTTGCATTCTCATCTTACACCAAACG-
EGHV5A/B L5-1 5'-GGGACGACGCAAGGTACGGTATAACCC-3'
Rd2A,2B,3A,3B (multiple alternative combinations)

SET 15/16A U71/gM specific for *EEHV3/4/7*

R1 LGH6793B 5’-GCCATCGGGATCCGGAAAARACGCC-3’
R2 LGH9209 5’-CCACCGATACTACATGGGAAACATAGCC-3’ (same as LGH9137)
L4 LGH9211 5'-GGCAACATCACCGTAATGTACGTGGTTTGG-3’
L2 LGH9210 5’CCAGTACGATAAGATCTACCTGGACGA-3’ (same as LGH9138)
L1 LGH6792B 5’-GGGCATCTTCTTCACCCTCATAGCCATC-3’
rd1 R1/L1 = 700-bp, rd2 R1/L2 = 670-bp; rd3 R2/L4 PCR = 640-bp.

SET 16D U71/gM specific for *EEHV3B* and *EEHV7A/B*

R3Bsp LGH9682 5’-GCCTTCGAGGAGCTATCGGATGAAGAGA-3’
L3Bsp LGH9684 5’-GAGGTACAAACCCTTGGTGGTGCTCACA-3’
R3COM LGH9685 5’CTRCTGGACGAC GAAGCCTTCGAGGAGC3’
R7COM LGH9686 5’-CTGTTGGACGACGACGCGTTCGAGGACC-3’
L3+7COM LGH9687 5’-GCGTTGCCTCMGGATTAYTAYCACAAC-3’
Lalt3COM LGH9210 =(9138) 5’-CCAGTACGATAAGATCTACCTGGACGA-3’
rd1 R3/L3+7 =560-bp, rd2 R3B/L3+7 =545-bp, R3B/L3B =510-bp.

SET 18C U73(OBP) specific for GC-rich *EEHV3/4/7*

R1 LGH9279 5’-CGAGCCCCTATGGGGGTCTGGNAAGAC-
R2 LGH9280 5’-GACSGCGGCYATGATCTCGTGGCTCA-
L4 LGH9283 5’-GGCACGAGGCGACGCACGCCAGGAACTTG-
L0 LGH9288 5’-GACTGGTATACTGACATCATGTCGGGACC-3’
rd1 R1/L0 =940-bp, rd2R1/L4 =360-bp, rd3 R2/L4 = 345-bp

SET 26 U48.5/U49 (gH-TK)

R1 LGH9985 5’CTGTTCCTCTGTCTCCACGCGCCTCAGCGT-3”
R2 LGH9986 5'-AGCATGGAGTACAACGGCATGTAGCTGA-3'
L2 LGH9987 5'-GTATAGGTGTAGGTAAGACGTCACTGTTC-3’
L1 LGH9988 5'-CTCACCGTTTACCTAGAAGGTTGTATAGGTG-3'
rd1 R1/L1 = 360-bp, rd2A R1/L2 = 300-bp, rd2B R2/L1 = 280-bp, rd3AB R2/L2= 250-bp

SET 26D new alternative specific for PAN *EEHV3/4/7* GC-branch

R1 LGH11242: 5’-CCTGGCGAAAGGACCAGAGCATGGAG-3’
R2 LGH11243: 5’-GCAGGTACTGGTGCAGGGGAAACAC-3’
L2 LGH11244: 5’-CTGGACCCAGTGGTTTCCCGAAAACG-3’
L1 LGH11245: 5’-CGTCACTGTTCAAATACGCCGCGGATAAC-3’
rd1 R1/L1= 375-bp, rd2A R2/L1 = 320-bp, rd 2B R1/L2 = 305bp, rd 3AB R2/L2 = 250-bp

SET 27 U81(UDG)

R1 LGH9989 5‘-AGGTAT TGGTTGGCTTTCACGAAGTG-3‘
R2 LGH9990 5‘-CTGGCGGCCGCCAGGGGGGAAGGGTGAGC-3‘
L2 LGH9992 5’-GCCGACGGCCTGGCCTTCTCCACGGGTGACGG-3’
L0 LGH11149 5‘-CGAAGAGTGGGTCGCCTTTCTAGA-3‘
rd 1R1/L0 = 520-bp, rd 2A R1/L2 = 435-bp, rd2B R2/L0= 470-bp, rd R2/L2 = 360-bp;

SET 28 E54(vOX2-1) (*EEHV1A/1B/6* only)

R1 5’ LGH837 5’- ATGCTTCAGAGAAAGTAAGGTAC-3’.
R2alt LGH132 5’-GGTCGTAACGCAAGATGAGCGAG-3’
L1 LGH8472 5’-GTGTTGCCGCCACGATGCTTCTACG-3’
rd1 R1/L1 = 910-bp; rd 2 R2/L1 = 740-bp

SET 29A U51(vGPCR1) specific for *EEHV3/7*

R1 LGH10784 5’-CGTTCCCGCTGTGGATCTACCAGGC-3’
R2 LGH10785 5’-CAGCGGGATGTTCTACACCATG-3’

R3 LGH10786 5'-GTCACCTTCGATCGCTGGTACTG-3'
L2 LGH10787 5'-GCTCTGTCCCGAGTACAGGTAGCAG-3'
L1 LGH10788 5'-CATGCCGTAGAACGGCCCTTGGG-3'
rd1 R1/L1 = 490bp, rd2A R1/L2 = 310bp, , rd2B R2/L1 = 420, rd3AB R3/L2 =<400bp

SET 30 E4(vGCNT1) specific for *EEHV4/7*

LGH10789 vGCNT1 EEHV7R1 5'-AGGGCCATCTACGCCCCGCAGAACCT-3'
LGH10790 vGCNT1 EEHV7 R2 5'-TCCTGGAGTCGCGTGCAGGCCGA-3'
LGH10791 vGCNT1 EEHV7 L2 5'-CATGTCCGAGGTGTCGTACTTG-3'
LGH10792 vGCNT1 EEHV7 L1 5'-TCGTAGTAGTGCCACTTGACGAACCTG-3'
rd1 R1/L1 = 660-bp rd2A R2/L1 = 515bp, rd2B R1/L2 = 525bp, rd3AB R2/L2 =415-bp

SET 35 U14 specific for *EEHV3*

LGHU14-R1 5'-GTCTTCGCAARCCGCGCTGGTCGGC-3'
LGHU14-R2 5'-GTCTCTGGATGTTCTGGGCATCCAG-3'
LGHU14-L2 5'-CCGTGTATGAARTACGTRTGTAAC-3'
LGHU14-L1 5'-CGAGCCGATATCGTCGACGATCTG-3'
rd1 R1/L1 = 920-bp, rd2A R1/L2 =870-bp, rd2B R2/L1 = 860-bp, rd3AB R2/L2 = 810-bp

SET 36 U42(MTA) specific for *EEHV3A/B* includes across ex1/ex2 boundary

LGHU42-R1 5'-GTAGTCGAAGACAGCGCGTTCTAG-3'
LGHU42-R2 5'-GACAACATGTTCGACACTCCGGTC-3'
LGHU42-L2 5'-GACGTTCGGCAGCTTTCCCGTGTA-3'
LGHU42-L1 5'-GACGAACTTTTTCTCCATGAGCATGG-3'
rd1 R1/L1 = 900bp, rd2A R1/L2 = 860bp, rd2B R1/L2 = 870- bp= rd3AB R2/L2 = 830-bp

SET 37 U43(PRI) specific for *EEHV3A/B*

LGHU43 R1 5'-CAGGTGCTCTTCGCGACCGAATAC-3'
LGHU43 R2 5'-TGCGGATGCGATGTTGACCACCT-3'
LGHU43 L2 5'-CAGAACGCTATGATCGTTTCGTC- 3'
LGHU43 L1 5'-CTGACGCGGGTAGAGTTTTCGCACG-3'
rd1 R1/L1 = 760bp, rd2A R1/L2 = 720bp, rd2B R2/L1 = 710bp, rd3AB R2/L2 = 670-bp

SET 38 U82(gL)-E37(ORF-Oex3) specific for *EEHV3/4/7*

LGH11558 New R1 5'-CGTTCACGGGCGGYGGGTASACGATC-3'
LGH11559 New R2 5'-GGGTASACCGATCTCGGAGACCCG-3'
LGH11560 New L5A 5'-GACGCGTTTATCGTCACRGGGAGGG-3'
LGH11561 New L4 5'-TTYGACACTCCCGCCCTCCTTGACG-3'
LGH11562 New R3 5'-CACYCTYAGTTCCTCSGTRTTATA C-3'
LGH11563 New R4 5'-GAA TAGAYTTAACRCCGGTAGGAAC-3'
LGH11564 NewL3 5'-GTTCCTACCGGYGGTGTTAAATCTATTC-3'
LGH11565 New L2 5'-CAGATGATAACGTCCCTSGACAAGTG-3'
LGH11566 New L1 5'-CAGATYACGTATGTYTTTRATCCG-3
RdA: rd1 R1/L4 =680-bp, rd2A R1/L5 =640-bp, rd2B R2/L4 =66-bp, rd3A R2/L5 =620-bp;
Rd2B: rd1 R3/L1=705bp, rd2A R3/L2 =610-bp, rd2B R4/L1 =565-bp, rd3A R3/L3 =535-bp,
rd3B R4/L2 =565-bp. Multiple combinations over 1800-bp.

RESULTS

Positive *EEHVs* DNA sequences were identified in all nodules from the five immobilized juvenile and sub-adult *Loxodonta africana* and in the lung from the just dead *Loxodonta africana*

in Kenya; and in four nodules from the juvenile male elephant in Botswana designated BWM1 and in a single nodule on a juvenile female calf designated BW2F; and in nodules from two immobilized sub-adult *Loxodonta africana* in South Africa designated RSA BussA, RSA BussB; and from ear wart-like lesions from an adult South African bull *Loxodonta africana* designated RSA WildSpirit. These were examined at up to 12 cPCR primer sets that detected known selected high GC-rich *EEHV3* or *EEHV7* loci, as well as several more generic cPCR primer sets that also detected *EEHV2* or *EEHV6*. Overall, these assays identified multiple examples of *EEHV2, EEHV3A, EEHV6, EEHV7A* and novel subtypes of *EEHV3B* and *EEHV7B* and rare subtypes *EEHV3C to EEHV3F* as well as multiple examples of *EGHVs*. (Genbank acc# KT832491.1 to KT832495.1). Nearly all of the nodule biopsies proved to contain multiple different virus types and strains, with multiple viral genomes frequently present within the same purified PCR bands. For example, the random lung biopsies from the wild *Loxodonta africana* in northern Kenya designated KENYA SAM6 and SAM7 yielded U77(HEL) DNA sequences of *EEHV2, EEHV3A, EEHV6* and *EEHV7A* in the same cPCR reaction [24]. For *EEHV3* especially (the most commonly encountered virus in the nodules), the large number of nucleotide variations found between the numerous different strains and patterns of subgrouping among them have often allowed us to designate more than just *EEHV3A* and *EEHV3B* and further define rare subtypes *EEHV3C, EEHV3D, EEHV3E* and *EEHV3F.* Curiously, in KENYA calfGM, we found no hints of any *EEHVs* strains other than one particular strain of *EEHV3A* being present in her two trunk nodules.

TABLE 1.

Elephant Herpesviruses Detected in *Loxodonta africana* Nodules, Lung and Tissue Biopsies

	EEHVs								***EGHVs***				
	2	**3A**	**3B**	**3C**	**3D**	**6**	**7A**	**7B**	**1B**	**2**	**3B**	**4B**	**5B**
KENYA calfMT Nod	2	3A	3B	3C			7A	7B	1B				
KENYA HIM Nod		3A	3B		3D		7A	7B	1B				
KENYA MMM Nod	2	3A	3B				7A	7B	1B	2			
KENYA MMF Nod		3A	3B	3C					1B	2	3B		
KENYA calfGM Nod		3A									3B		
KENYA SAM6,7 Lung	2	3A				6	7A		1B		3B	4B	
Botswana BW1M Nods		3A	3B		3D		7A		1B	2			
Botswana BW2F Nod		3A					7A		1B	2			
RSA WildSpirit Ear Wart			3B								3B		
RSA BussA Nod	2		3B				7A			2			5B
RSA BussB Nod			3B	3C						2			5B
USA NAG31 Ear lesions, organs			3B										
England Howletts Trunk Nod													5B

Saliva from Kenya and Botswana mother plus calf pairs and from 28 additional *Loxodonta africana* in Botswana, Kenya and South Africa were screened with up to seven selected GC-rich branch cPCR loci (POL, HEL, U71/gM, OBP, TK, vGCNT1 and UDG) yielding more than 75 positive unambiguous *EEHVs* DNA sequences of *EEHV2, EEHV3A, EEHV3B, EEHV3C, EEHV 3D, EEHV6, EEHV7A* and *EEHV7B* and an additional two dozen unresolvable mixtures. Overall, the predominant result was for an *EEHV3A* strain (26 examples), whereas *EEHV3B* strains were detected eight times, *EEHV2* strains three times (just with the POL primers) and

EEHV7A three times (HEL or U71/gM.) Saliva from some mother/calf pairs were identical, but more often were different. Saliva swab samples from four of the same Kenya juveniles with nodules also detected between one and four subtypes of *EEHV3A, EEHV3B, EEHV3C, EEHV3D* and one or two subtypes of *EEHV7A* and *EEHV7B* as well as *EGHV1B* and/or *EGHV3B* each, some of which matched the strains in the skin nodules but mostly did not.

Saliva swabs from 25 *Loxodonta cyclotis* were screened by cPCR sequencing with more than six GC-rich branch *EEHVs* primer sets including POL, HEL, TER, UDG, TK and OBP loci. Over half of 25 saliva samples from *Loxodonta cyclotis* in Gabon were positive for at least one *EEHV3* or *EEHV7* strain, and once for a novel *EEHV6*, but no *EEHV2* was detected, and many of these were also present as unusual rare variants of non-A/non B Subtypes of *EEHVs* that we found in nodules and saliva from *Loxodonta africana.* Unusually, unlike the results from the nodules, only a single *EEHV* strain was detected in any one saliva sample. The high prevalence of *EEHV3B* suggests that most likely *EEHV3B* and the more rare *EEHV3C, EEHV3D, EEHV3E, EEHV3F* subtypes have been exchanged and recombined during overlapping range interactions between *Loxodonta africana* and *Loxodonta cyclotis* hosts.

We carried out extensive cPCR with generic POL and new alternative second and third round primer sets targeting specifically *EGHV1B, EGHV2, EGHV3B, EGHV4B* and *EGV5B* to screen nodule biopsies and saliva from the same *Loxodonta africana* and *Loxodonta cyclotis* that we screened for *EEHVs.* Overall, nodule biopsies from six of the seven wild *Loxodonta africana,* the KENYA SAM6/SAM7 lung biopsies, RSA Wild Spirit (SAG7) non-nodular ear wart biopsy and more than half of the saliva samples from 28 wild *Loxodonta africana* and 25 *Loxodonta cyclotis* proved positive for clean unambiguous detection of at least one type of *EGHVs*, although many more clearly contained unresolvable mixture of multiple *EGHVs* genome types.

TABLE 2.

Elephant Herpesviruses Detected in saliva from
Loxodonta africana and Loxodonta cyclotis
AFRICA

	EEHVs	***EGHVs (1B* variants: '=TTTT, "=CCGC)**
Kenya MT	7A	3B
Kenya cMT	3A 3B 3D 7A	1B' 3B
Kenya MMF	3A 3C 7A 7B	3B
Kenya cGM	3A 7A 7B	3B
Kenya mGM	3A 3B 3D	
Kenya mMMM	3B 7B	
Kenya MMM	3B	2 3B
Kenya Honesty	7A	
Kenya Taurus	7A	
Kenya Mercury		3B 5B
Kenya Resilience		3B
Botswana BW1 M	3A 3B 7A.	1B' 2
Botswana BW2	3A	1B'
Botswana BWAbu Sirini	3A	
Botswana BWAbu Lerato	3A 3B 7A	
Botswana BWAbu Paseka	2 3A 3B	
Botswana BWAbu Kitti	2 3A 7A	
Botswana BWChobe3	3A	1B'
Botswana BWChobe4	7A	1B'
Botswana BWLA2 Doji	2 3A 3B 3C	
Botswana BWLA6 Jumbo	3A 3B	
Botswana BWLA9 Pfumo	3A 3B	
Botswana BWLA4 Tembe	3A	
Botswana BWLA1 Janai	3B	
Botswana BWLA7 Tendai	3A	
South Africa RSA Wild Spirit		1B' 3B
South Africa RSA Matambu		1B''. 3B
South Africa RSA Proud		1B'
South Africa RSA Buss 3	3A 3B	
GABON GA1 to GA25 (*L. cyclotis*)	**3A 3B 3C,3D,3F 6 7A 7B**	**1B'' 2 3B**

Our sequential saliva study of seven *Loxodonta africana* at Six Flags Safari Park showed that *EEHV2, EEHV3A, EEHV3B, EEHV6, EEHV7A* and *EEHV7B* and multiple *EGHVs* are periodically shed in saliva, with usually only one virus being detectable at a time. Saliva from two adult females, designated USA SFJ Joyce and USA SFL Lucy, were collected bi-weekly over a full 12-month time period from January, 2012 through January, 2013 for a total of 47 samples each. Joyce, estimated year of birth 1980, was among a group of 99 juvenile *Loxodonta africana* imported from Zimbabwe to Florida in 1982 to 1984 [2]. VR Pearson could find no documentation among the records provided by Jacobson as to whether Joyce may have had any visible trunk nodules at that time, and since then Joyce had lived at several locations across USA before settling in 2010 at Six Flags Safari Park, where she has resided ever since. Lucy, estimated year of birth 1969, had been imported from Uganda directly to Six Flags Safari Park in 1970 and has remained at the park since. The other five herd members, females designated USA SFT Tanya, USA SFG Gloria, USA SFS Sheila, USA SFB Barbara, USA SFD Debbie, had been imported from Uganda at the same time as Lucy and had never left Six Flags Safari Park. These elephants were sampled by saliva swabs intermittently for a total of fifteen times from 2012 to 2019.

EEHV2, EEHV3A, EEHV3B and *EEHV6* were all detected in the saliva swabs from both elephants Joyce and Lucy at various time points, but with different time-course shedding patterns. Overall, Joyce scored positive for *EEHV2* in 17 of 47 samples. Only a single sample from Joyce, *EEHV2* POL in collection #4, January 2012, gave a band of sufficient intensity to obtain clear unambiguous DNA sequence data directly from the first round cPCR amplification. All others required second or third round amplification first. Lucy was somewhat similar proving positive for *EEHV2* in 8 of 47 samples. In contrast, Joyce was positive for *EEHV6* in 19 of 47 samples and Lucy was positive for *EEHV6* in 6 of 47. For *EEHV3,* both Joyce and Lucy shed both *EEHV3A* and *EEHV3B* intermittently for a total of Joyce 11 of 47 and Lucy 10 of 47. Joyce was positive four times for four different viruses at the same time (*EEHV2, EEHV3A, EEHV3B* and *EEHV6*) and Lucy was positive at some point for two or three different *EEHV* species at the same time (either *EEHV2, EEHV3B* and *EEHV6* or *EEHV2, EEHV3B*). We interpret that these assays detect evidence of previous persistent infections rather than necessarily currently active systemic infections or shedding as might be detected at high levels in blood or trunk wash samples from *EHD* cases.

TABLE 3.

Elephant Herpesviruses Detected in saliva from *Loxodonta africana*

SIX FLAGS SAFARI PARK, USA

	EEHVs						***EGHVs***		
USA SFJ (Joyce)*	2	3A	3B	6			1B’		
USA SFL (Lucy)**	2	3A	3B	6			1B’	2	3B
USA SFT (Tanya)**	2	3A	3B	6	7A		1B’’		
USA SFG (Gloria)**	2	3A	3B	6			1B’		3B
USA SFS (Sheila)**	2	3A	3B		7A	7B (novel)	1B’		3B 4B
USA SFD (Debbie)**	2	3A	3B				1B’’		
USA SFB (Barbara)**	2	3A	3B				1B’		

*born in Zimbabwe
**born in Uganda

EGHV1B variants:
‘=TTTT, ”=CCGC

Sixteen examples of *EGHV1B* (the most abundant *EGHVs* species we encountered) were detected within sub-Saharan Africa, including two invariant polymorphism patterns that differ by only 4/450-bp (0.9%). The more commonly found TTTT pattern was seen in 13 of 14 EGHV1B cases from *Loxodonta africana* within Africa, and the more rare CCGC pattern was found only once in *Loxodonta africana* adult bull Matambu (SAG5) (Timbavati, South Africa) in continental Africa. Three *EGHV1B* positive samples from *Loxodonta cyclotis* fall into the same CCGC variant. These same two polymorphic variants were detected in three whole herd saliva sets from Six Flags Safari Park. USA SFJ Joyce, SFL Lucy, SFG Gloria, SFS Sheila and SFB Barbara carried the *EGHV1B* TTTT variant whereas USA SFT Tanya and SFD Debbie carried the CCGC variant identical to South African RSA Matambu (SAG5) as noted by asterisk* in TABLE 2. The USA SFJ Joyce saliva sample #97 collected on November 21.2012 has been designated the prototype for *EGHV1B*, NAG9, Genbank #KC810011.1. In saliva samples from

additional USA *Loxodonta africana* (not shown in Table 3) we only found the CCGC variant in less than one-quarter of the samples tested.

The DNA sequences for the *EGHV2* POL locus detected in the wild *Loxodonta africana* (RSA BussA, KENYA MMM, KENYA MMF, BWM1, BWF2 and in the Six Flags Safari Park USA SFJ Joyce and USA SFT Tanya are identical to those *EGHV2* sequences we identified and reported in the nine month sequential saliva collection study of *EEHV3B* survivor *Loxodonta africana* NAG13 Samson, Genbank acc# KU726836, # KU726837,[23] and were just one to four bp different from the prototype *Elephas maximus* EGHV2 POL [20].

EGHV3B was detected frequently in skin nodules, ear warts and saliva from *Loxodonta africana* and in saliva from *Loxodonta cyclotis*. Quite unexpectedly however, we detected widespread presence of *EGHV3B* in necropsy organs from an adult female *Loxodonta africana,* NAG31, in a USA zoo, including in ear lesions, heart, lymph node salivary gland, serum and thymus. We have rarely detected any *EGHVs* in any internal organ tissues from *EHD* cases or necropsy samples in USA zoos.

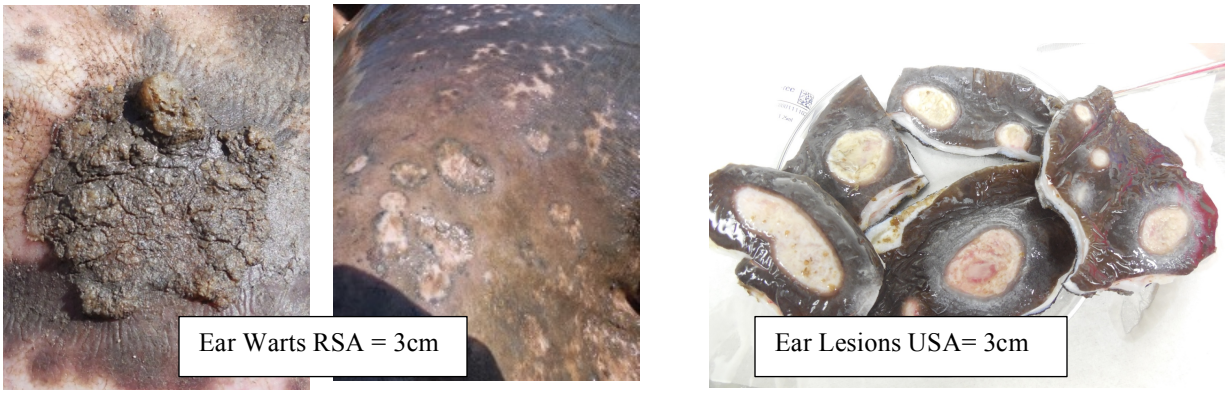

FIGURE 12. Left, close up of ear warts on back of ear, from immobilized adult bull *Loxodonta africana* RSA Wild Spirit, Balule, South Africa. 6mm biopsies were collected by VR Pearson and preserved in Qiagen RNAlater.
FIGURE 13. Middle, back of ear showing multiple lesions on RSA Wild Spirit. cPCR showed ear warts were positive for *EEHV3B* and *EGHV3B. Copyright VR Pearson, 2013.*
FIGURE 14. Right, ear lesions on back of ear from necropsy samples of adult female *Loxodonta africana* NAG31 in USA zoo, received by VR Pearson 2014. Ear lesions, heart, lymph node salivary gland, serum and thymus, were positive for *EGHV3B* only, no *EEHVs* were detected. *Copyright VR Pearson, 2014.*

We detected *EGHV4* in wild *Loxodonta africana* lung biopsies designated KENYA KG5 (from the same lung biopsies designated KENYA Sam6 and Sam7, and in saliva from USA SFS Sheila NAG24, and in Samson NAG17, Genbank acc.#KU726840.[23]. Subsequently, we discovered an evidently novel Asian elephant version of *EGHV4* in spleen tissue from a three year old male captive-born *Elephas maximus* NAG72 in New Mexico, USA, that died suddenly of *EHD*, Genbank Acc. #PQ379918. Identical 250 bp sequences were obtained from saliva from a captive-born living male juvenile *Elephas maximus* NAG71 in Texas and in the spleen from a deceased elderly USA *Elephas maximus* NAG56 in Florida, USA. These results led us to

reassign all the previous *African Elephant EGHV4s* and Asian Elephant *EGHV4s* as distinct subspecies *EGHV4A* or *EGHV4B* endogenous to either *Elephas* or *Loxodonta* respectively.

We detected *EGHV5B* in skin nodule biopsies from two immobilized sub-adult *Loxodonta africana* in Kruger National Park, South Africa: BussA SAG3, BussB SAG4, and in saliva from an adult female *Loxodonta africana* in Kenya designated KENYA Mercury KG16. In this case, the 450-bp DNA sequences obtained after second round PCR with the generic Pan-EGHV primers were identical to one another and to those from the prototype NAG8 *EGHV5B* (Genbank accession #JX268522). Most recently, Sanger sequencing from first round cPCR using *EGHV* PAN POL primers R1/L1 of a trunk nodule biopsy from a 3 year old captive-born *Loxodonta africana* in England (biopsy collected by J Hopper, DVM, Howletts Wild Animal Trust, England, 2024) yielded a 439 base pair sequence of *EGHV5B,* Genbank Acc# PQ3979917, that is identical to the prototype NAG8. This is the first time we have been able to compare *EGHV* sequence results from a trunk nodule on a *Loxodonta africana* residing outside of Africa to nodule biopsies and saliva collected by VR Pearson in Botswana and Kenya. The nodule biopsy remains in England and has not been tested with extensive *EEHVs* primers or for *African Elephant Polyomavirus AelPyV-1* discussed below.

FIGURE 15.Trunk nodule on 3 year old *Africana loxodonta*, positive for *EGHV5*, photo courtesy J. Hopper, DVM, Howletts Wild Animal Trust; DNA extraction, cPCR and Sanger sequencing provided by A. Dasjerti, DVM, APHA, UK, for Howletts Wild Animal Trust, England, 2024.

DISCUSSION

While more than two dozen cases of severe or lethal *EHD* have been reported in African elephants in zoos in USA, Europe and Thailand, as yet no cases of lethal EHD have been documented in continental Africa, perhaps because the common presumption of anthrax poisoning precludes opening of carcasses for necropsy [25-29]. We expect cases of lethal *EHD* will be identified in wild *Loxodonta africana* and *Loxodonta cyclotis* as African veterinarians and pathologists begin to routinely include cPCR using *EEHVs*-specific primers to investigate sudden unexplained hemorrhagic deaths, as is routine necropsy protocol in Asian elephant range countries which has provided genetic confirmation of more than 125 cases of *EHD* mortality in

wild and captive *Elephas maximus, E.m sumatranus* and *E.m borneensis* across Asia and in zoos worldwide. [30-43].

Each of the *African Elephant Endotheliotropic Herpesvirus EEHV2, EEHV3, EEHV6* and *EEHV7* displays A plus B subtypes that may contain up to ten localized non-adjacent chimeric domains (CD) or hypervariable gene blocks encompassing anywhere up to seven distinct subtype clusters with large (15 to 50%) protein level differences. *EEHV3* strains fall into two major chimeric subtype clusters similar to those described previously in Asian elephants as *EEHV1AB, EEHV4A/B* and *EEHV5A/B* [44,45]. In *EEHV3A* strains only, parts of the CDs including vECTL, gB. gH, TK, vGPCR1, gL and UDG can instead have several other alternative highly diverged but unlinked subtype clusters (designated *EEHV3C*, *EEHV3D, EEHV3E,* and *EEHV3F,* Genbank acc.# MN373268, NC_077039, NAP42 EEHV7A Genbank acc# KU321582, NAP62 Samson EEHV3B KT832467.1-KT832476.1, KT832491.1-KT832495.1 [29]. These differ greatly (by between 15 and 45%) in several linked but non-adjacent chimeric domains (CD-I, CD-II, CD-III, and CD-V) totaling 15 to 20-kb in size, but by just 2 - 3% everywhere else. Based largely on the summed higher levels of individual protein divergence across the whole *EEHV3A* versus *EEHV3B* genome as well as the relatively high frequency of *EEHV3B* being found in saliva swabs from Gabon, compared to *EEHV3A* being far more prevalent in Botswana, Kenya and South Africa we judge that these two *EEHV3* groups should in fact be designated as distinct species with *EEHV3A* likely having evolved in *Loxodonta africana* and with *EEHV3B* probably having evolved originally in *Loxodonta cyclotis.* Our ongoing genetic evaluation of the few recent *EHD* cases attributable to *EEHV2* and *EEHV6* suggest that similar division into A and B subtypes may be appropriate, as found in *EEHV3A/3B* and *EEHV7A/7B* pairs that differ by 7 - 9% overall with many other genes not just the CDs varying significantly.

The five *EGHV* POL genes found in *Loxodonta* and *Elephas* are widely diverged displaying between 37 and 45% nucleotide polymorphisms and fall into three ancient deep lineages within the mammalian gammaherpesviruses corresponding to estimated last common ancestors (LCA) occurring about 200 million years ago [20]. Four of the five known *EGHV* species: *EGHV1, EGHV3, EGHV4* and *EGHV5* (but not *EGHV2*) divide into consistent A and B diasporas that differ by around 3 - 5% (15 to 24/450-bp polymorphisms) and are largely specific for *Elephas* or *Loxodonta* respectively.

In a previous study conducted by VR Pearson, nodules biopsies from six of the seven wild *Loxodonta africana* in Botswana and Kenya, which were positive for one or more species of *EEHVs* and *EGHVs* as part this study, and a set of 30 year-old formalin-fixed paraffin-embedded (FFPE) trunk nodule biopsies collected by Jacobson from nine Zimbabwe-born elephants [2], were found to contain multiple genes of *African Elephant Polyomavirus (AelPyV-1)* [46, 47]. In two FFPE blocks from the same elephant, one #84-133 contained a skin nodule while the second #84-133A contained a lung biopsy as shown in FIGURE 16. Only DNA extracted from the skin nodule was positive for *AelPyV-1*. Additionally, saliva samples from 57 wild and 26 USA *Loxodonta africana* and 41 USA *Elephas maximus*; and from blood, serum, non-nodular skin warts, and internal organ biopsies from 2 wild and 5 USA *Loxodonta africana* and 6 USA *Elephas maximus* in our biorepository that previously tested positive for at least one species or subtype of *EEHVs* or *EGHVs* were screened for *AelPyV-1.* Sanger sequencing detected short

sequences of *AelpyV-1* large T antigen gene in only 8 out of 124 saliva samples and in no blood, serum, non-nodular skin warts, or internal organs. This suggests that *AelPyV-1* is generally restricted to skin-associated nodules. Further investigation is needed to ascertain whether *AelPyV-1* or infiltrating lymphocytes latently infected with EEHVs/EGHVs may be may be responsible for the raised proliferative trunk nodules on African elephants.

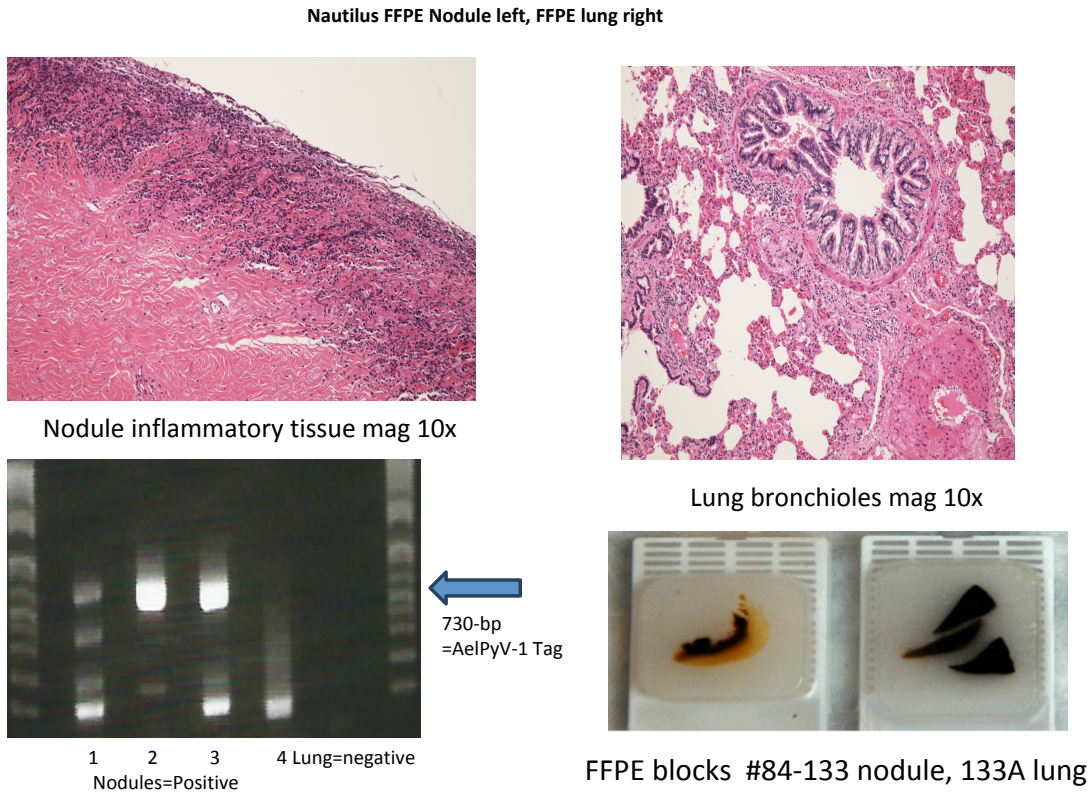

FIGURE 16. Nautilus *Loxodonta africana* FFPE #84-133 nodule top left, #84-133A lung top right.Fresh FFPE cassettes were made from thirty year old FFPE blocks, the 5 micron slices cut and stained with H & E. prepared by Catherine Reimmer and Kathy Q Cia, Histology Lab, Fox Chase Cancer Center, cPCR and diagnostic electrophoresis gel by VR Pearson, 2019.

CONCLUSION

We conclude that *EEHV2, EEHV3A, EEHV3B,* rare subtypes *EEHV3C-EEHV3H, EEHV6, EEHV7A* and *EEHV7B,* and *EGHV1B, EGHV2, EGHV3B, EGHV4B and EGHV5B* are endogenous viruses of *Loxodonta africana* and *Loxodonta cyclotis.* Our extensive library of elephant viral and genomic DNA samples are invaluable assets for development of vaccines targeting the pathogenic species of *EEHVs* afflicting elephants and for determining management plans for preserving genomic diversity in rapidly declining wild elephant populations in situ and isolated herds managed in preserves and zoos.

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Sample Collection and DNA Extraction: VR Pearson
Conventional PCR and isolation of purified DNA bands after gel electrophoresis: VR Pearson
Design of numerous *EEHV* species and gene locus specific primers: GS Hayward
Sequencing Analysis: VR Pearson and GS Hayward
Original manuscript draft: VR Pearson
Review & editing: VR Pearson and GS Hayward

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