

ELEPHANT ENDOTHELIOTROPIC HERPESVIRUS RESEARCH AND TISSUE REQUEST PROTOCOL

(Elephas maximus and Loxodonta africana)

Updated August 2010



ASSOCIATION
OF ZOOS &
AQUARIUMS

TABLE OF CONTENTS

Introduction..... 3

Elephant Endotheliotropic Herpesvirus Alert..... 3

EEHV Summary Points 5

All Facilities Must Help Find Answers to EEHV 6

Checklist for EEHV Samples 7

EEHV Necropsy Protocol..... 10

Research Requests and Contact Information..... 11

Logistics and Necropsy Tips 15

Elephant Necropsy Protocol Gross Examination Worksheet 16

Tissue Checklist..... 19

Consent Form for Use of Samples by AZA Elephant TAG/SSP 20

Frequently Asked Questions about EEHV 21



INTRODUCTION

This protocol is a collaborative effort of the Association of Zoos & Aquariums (AZA) Elephant Taxon Advisory Group/Species Survival Plan (TAG/SSP) and the International Elephant Foundation (IEF). Its purpose is to provide a format for the systematic collection of information and samples that will add to our knowledge of Elephant Endotheliotropic Herpesvirus (EEHV) and contribute to the diagnosis and treatment of EEHV. All North American institutions caring for elephants will receive a copy of this protocol.

ELEPHANT ENDOTHELIO TROPIC HERPESVIRUS ALERT

Infectious disease is one of the factors threatening the long-term survival of Asian and African elephants. EEHV can be a fatal disease of elephants in human care and in the wild, and is one of the many conditions which can impact the overall health and survivability of elephants. Young elephants are most vulnerable to EEHV, making it a particularly devastating disease. Reproductive failures and early deaths of juvenile elephants in North America and Europe have been attributed to EEHV, and EEHV has been confirmed as the cause of death in up to ten wild elephants in India, Thailand and Cambodia including both orphaned and free-ranging calves [Reid et al 2006; Zachariah et al, IEF Conservation & Research Symposium conference abstracts Bangkok 2008, Pretoria 2010]. It is not known if there have been widespread outbreaks in Asia; however the impact of EEHV may now be exacerbated by increased fragmentation of elephant populations. Little is known regarding basic epidemiology of this virus, such as transmission patterns, incubation period, site, and cell tropism for viral latency.

EEHV is associated with a group of unique herpesviruses (8 species or sub-species - EEHV1A, EEHV1B, EEHV2, EEHV3, EEHV4, EEHV5, EEHV6 and EEHV7 - of which 5 have caused fatal disease [Ossent 1990, Richman 1999; Richman, 2000; Garner, 2009; Latimer 2010]. These herpesviruses affect primarily young elephants (<10 years of age) and can have a fatal outcome. The onset of the disease may be very rapid with few prodromal signs and peracute death within hours to 7 days. Clinical signs are often vague and can include lethargy, lameness, colic, anemia, thrombocytopenia, edematous swellings of the head and thoracic limbs, oral ulceration and cyanosis of the tongue. Necropsy findings are consistent with vasculitis and include extensive cardiac and serosal hemorrhages and edema, hydropericardium, cyanosis of the tongue and oral and intestinal ulcers. Histological features are microhemorrhages with very mild inflammation in the heart, liver and tongue accompanied by intranuclear inclusion bodies in the capillary endothelium. Transmission electron microscopy of the inclusion bodies shows 80-90 nm diameter viral capsids consistent with herpesvirus morphology.

As of August 2010, there have been 39 known clinical cases in North America since 1977 with 29 deaths (27 in Asian elephants). EEHV1A is the most common type (21 deaths in North America) and there are significant differences even among the 21 EEHV1As. There have been four deaths worldwide from EEHV1B, two deaths from EEHV2, and one death each from EEHV3, EEHV4 [Latimer, 2010]. Diagnosis of EEHV is made by detecting herpesvirus DNA in EDTA whole blood using polymerase chain reaction (PCR). Of 20 sick calves that were treated with famciclovir, eight survived. Ganciclovir has also been more recently used successfully.

Serological tests have been developed to detect antibodies to EEHV1A in Asian elephants. However, diagnostic tests are confounded by the inability as yet to cultivate any of these viruses *in vitro*. At present about 10% of the Asian elephants tested in the US have given consistently positive serological results; these animals are predominantly greater than 30 years old and were wild-born. Therefore, it is likely that many of the wild-born elephants in the North American population were carrying EEHV1 strains upon importation, suggesting that there may be asymptomatic carriers among North American elephants. The serological status of North American African elephants has yet to be investigated. A pilot trunk wash study has shown low level viral DNA shedding in two asymptomatic adults with the same strain of EEHV1 that caused the death of a calf two years earlier at the same facility [Stanton, 2010].

Herpesviruses have been evolving within most mammalian host species for over 300 million years, where they usually establish a stable host-parasite relationship that only rarely leads to serious or fatal disease. Many animals, including humans, carry several species of herpesviruses throughout their lives and never become clinically ill. Once inside a host animal, herpesviruses establish a latent (or hidden) phase after causing mild symptoms or asymptomatic infection. The virus then persists in the body, undetected by diagnostic tests or the body's immune system. For transmission to a new host, all herpesviruses need to have a mechanism by which they occasionally reactivate and shed infectious particles from localized skin lesions or in saliva or other body fluids. Different herpesvirus families establish latency in different cell types or organs and have different mechanisms for reactivation. For reasons not completely understood, some primary or reactivated herpesvirus infections lead to massive viremia, where virus particles circulate through the bloodstream, infect multiple organs and cause serious or lethal systemic disease.

Under normal conditions, primary asymptomatic infections with endogenous herpesviruses should be nearly universal in early infancy in the natural well-adapted host species. While serious disease is not normal in the natural host species for most herpesviruses, serious disease can occur if the host species is immunosuppressed, fighting other concurrent infections, or in rare situations when a virus comes into contact with and is able to infect an animal that is not the normal host species. Healthy adult African elephants carry EEHV2, EEHV3 and EEHV6 in lymphoid lung nodules, where it can be detected because of localized reactivation in epithelial cells. A few African elephant calves have also been reported to have EEHV1 in skin nodules. Although studies have not been performed to verify this hypothesis, it is likely that many healthy wild-born Asian elephants are asymptotically infected as well. There is no treatment for latent herpesviruses in animals or humans: however anti-viral drugs can suppress viral replication and cell damage when virus is circulating. It is believed that early detection of EEHV and immediate intervention with supportive care are critical to the success of treating an elephant affected by EEHV. Antiviral medications may also play an important role in treatment. Timely intervention with the human anti-viral drug famciclovir is credited with contributing to the survival of eight Asian elephant calves with confirmed EEHV disease. No animals are known to have survived systemic EEHV disease without treatment; however, treatment does not guarantee recovery.

EEHV infections in elephant populations in human care may be a potential useful predictor for EEHV's impact on the increasing small, isolated wild elephant populations in Asia. Plans to develop additional trunk-wash and serological assays specific for each of the other seven EEHV species based on the limited DNA sequence available have been initiated.

EEHV SUMMARY POINTS

- EEHV infection can be a fatal disease of African and Asian elephants and has been found in captive and wild Asian elephants.
- EEHV affects mainly young elephants (<10 years of age, peak between 1 and 3 years).
- Clinical signs are often vague and may include lethargy, lameness, colic, anemia, thrombocytopenia, edematous swellings of the head and thoracic limbs, oral ulceration and cyanosis of the tongue. Signs may progress to death within hours or days.
- Necropsy findings may include extensive cardiac and serosal hemorrhages and edema, hydropericardium, cyanosis of the tongue, oral and intestinal ulcers, and lymphoid nodules (3-30 mm) in lungs, skin and vestibule.
- Histological features are microhemorrhages in the heart, liver and tongue accompanied by intranuclear inclusion bodies in the capillary endothelium.
- 39 known clinical cases in North America since 1977 with 29 deaths (27 in Asian elephants). EEHV1A is the most common type (21 deaths in North America) and there are significant differences even among the 21 EEHV1As. There have been four deaths worldwide from EEHV1B, two deaths from EEHV2, and one death each from EEHV3, EEHV4 [Latimer, 2010].
- Diagnosis and status of EEHV in clinical cases is made by detecting herpesvirus DNA in EDTA whole blood and sometimes serum, using polymerase chain reaction (PCR).
- It is believed that early detection of EEHV and immediate intervention with supportive care and antiviral therapy are critical to the success of treating an elephant affected by EEHV.
- Famciclovir and ganciclovir have been used for successful treatment in elephants.
- Recent evidence shows that there are asymptomatic carriers among North American Asian elephants.
- Serological tests have been developed to detect antibodies to EEHV1A in Asian elephants. At present about 10% of the Asian elephants tested in the US have given consistently positive serological results; these animals are predominantly greater than 30 years old and were wild-born. The serological status of North American African elephants has yet to be investigated.
- Studies suggest that it is likely that many wild-born elephants in the North American population were carrying EEHV1 strains upon importation
- **There is no evidence of shedding of virus in semen or transmission of EEHV through breeding, natural or artificial insemination, or through transport. Therefore, the AZA Elephant TAG/SSP recommends that institutions continue to exchange elephants and elephant semen as specified in the breeding recommendations.**

ALL FACILITIES MUST HELP FIND ANSWERS TO EEHV

The knowledge we have gained and will continue to gain from the elephants held in North America is highly significant for the protection of elephant populations worldwide. There is still much that needs to be done to enable us to be able to prevent and treat this deadly disease in elephants.

In particular, we need each facility to:

- 1) Review this protocol with keepers and vets annually;**
- 2) Familiarize keepers and vets with EEHV, its symptoms, and research sample needs from healthy, sick and recently deceased elephants;**
- 3) Provide samples from each of your living elephants for ongoing research projects;**
- 4) Contact research groups at the first sign of any elephant injury or illness, regardless of how insignificant it might appear or whether or not a diagnosis of another issue has been made;**
- 5) Refer back to this protocol to determine samples needed from elephants under veterinary care;**
- 6) Contact research groups if an elephant is to be euthanized;**
- 7) Contact research groups immediately upon all elephant deaths;**
- 8) Identify necropsy team prior to elephant death and illness and provide them with a copy of this protocol;**
- 9) Develop an institutional EEHV diagnostic and therapeutic plan, especially for breeding facilities or those with young animals;**
- 10) Keep Elephant TAG/SSP chair and advisors informed of cases/suspects, deaths and planned euthanasias; and**
- 11) Contact the Elephant TAG/SSP chair and advisors if there are any questions or if more information is needed.**



CHECKLIST FOR EEHV SAMPLES

KEY

HIGH PRIORITY

* after participation in the 2-year EEHV serology study of preferably weekly sampling an alternate collection protocol is provided.

** anytime elephant exhibits any abnormal behavior, something as minor as not napping as long as normal to lameness, stiffness, lethargy, in appetite and the more common symptoms of EEHV

*** samples will be important should EEHV be confirmed.

**** urine and saliva may or may not be useful prior to treatment – yet to be determined.

	SAMPLES NEEDED BY CORNELL UNIVERSITY	SAMPLES NEEDED BY NATIONAL ELEPHANT HERPESVIRUS LAB (NEHL)	SAMPLES NEEDED BY JOHNS HOPKINS UNIVERSITY	SAMPLES NEEDED BY BAYLOR UNIVERSITY AND/OR HOUSTON ZOO	PRINCETON UNIVERSITY
Healthy adult Asian elephant	EDTA WB from known survivors or known herds with a high incidence of EEHV	EDTA WB, serum weekly then quarterly *		Trunk wash sample	
Healthy Asian elephant calf	EDTA WB from known survivors or calves in high risk situation - herds with a high incidence of EEHV	EDTA WB, serum weekly then quarterly *		Trunk wash sample	
Healthy adult African elephant	EDTA WB from known survivors or known herds with a high incidence of EEHV	EDTA WB, serum weekly then quarterly *		Trunk wash sample	
Healthy African elephant calf	EDTA WB from known survivors or calves in high risk situation - herds with a high incidence of EEHV	EDTA WB, serum weekly then quarterly *		Trunk wash sample	
Birth of Asian calf	Umbilical cord and blood	Placenta, EDTA WB and serum from mother and calf	Umbilical cord and blood		Amniotic sac
Birth of African calf	Umbilical cord and blood	Placenta, EDTA WB and serum from mother and calf	Umbilical cord and blood		Amniotic sac

	SAMPLES NEEDED BY CORNELL UNIVERSITY	SAMPLES NEEDED BY NATIONAL ELEPHANT HERPESVIRUS LAB (NEHL)	SAMPLES NEEDED BY JOHNS HOPKINS UNIVERSITY	SAMPLES NEEDED BY BAYLOR UNIVERSITY AND/OR HOUSTON ZOO	PRINCETON UNIVERSITY
Unconfirmed illness**	EDTA WB	EDTA WB, serum, plus swabs/biopsies if lesions present. Also bank EDTA WB and serum from every member of herd and additional EDTA blood and serum from case***			
Confirmed illness prior to treatment	EDTA WB	EDTA WB, serum, saliva, urine**** from case and every member of herd, banked EDTA WB and serum from case and every member of herd up to 3 weeks prior to diagnosis.	2-4 ml EDTA WB and 20-100ml serum, to be shipped unfrozen for virus genome sequencing and virus cell culture	10 – 100 ml of serum frozen and sent on dry ice to Baylor University and Houston Zoo, for viral sequencing	
Confirmed illness after treatment	EDTA WB	EDTA WB, serum, from case and every member of herd	1-2ml EDTA WB and 1-2ml serum	10 – 100 ml of serum frozen and sent on dry ice to Baylor University and Houston Zoo, for viral sequencing	
Necropsy tissue from EEHV death in Asian or African elephant	EDTA WB, heart, liver, lung, spleen, tongue, skeletal muscle, brain, pericardial and peritoneal fluid.	Tongue, heart, liver, spleen, intestine, WB, serum, any tissue with a lot of hemorrhages. Any kind of nodules – skin, vestibular, lung etc.	EDTA WB, heart, liver, lung, spleen, tongue tissue samples unfrozen on ice for virus cell culture.		Heart, bone marrow, intact salivary gland, trigeminal ganglion
Necropsy tissue from non-EEHV death in Asian or African elephant	2 sets of tissues for “normal” reference	Tongue, heart, liver, spleen, WB, serum. Any kind of nodules – skin, vestibular etc. hilar, mandibular, thoracic and mesenteric lymph nodes.	Latent EEHV detection: Look hard for hidden palpable "herpes" lung lymphoid nodules (likely few, white to gray, smooth or spongy texture, small 2-10 mm in diameter-will likely need to bread loaf the lung to locate).		Aorta, heart, trachea,

	SAMPLES NEEDED BY CORNELL UNIVERSITY	SAMPLES NEEDED BY NATIONAL ELEPHANT HERPESVIRUS LAB (NEHL)	SAMPLES NEEDED BY JOHNS HOPKINS UNIVERSITY	SAMPLES NEEDED BY BAYLOR UNIVERSITY AND/OR HOUSTON ZOO	PRINCETON UNIVERSITY
Seropositive Elephants		Periodic EDTA WB, serum, saliva, trunk washes, urine.			
Other samples		Semen aliquot each time collected			



EEHV NECROPSY PROTOCOL

We hope that institutions will not have to face the immense task of performing an elephant necropsy, but if this occurs, it should be viewed as an important learning opportunity. **Collection and review of the requested data and samples is our best means of defeating EEHV.** With the increased availability of digital cameras, it is strongly recommended that photographs of both normal and pathologic structures be recorded for future reference.

Specific information about EEHV sample and data collection during an elephant necropsy is included in this protocol. Please send the completed forms to Dr. Michele Miller (contact information below).

Broader necropsy information and requests for samples for other research projects, in addition to EEHV, are contained in a separate document, Elephant Necropsy Protocol available online at www.elephanttag.org/Professional/ElephNecropsy_2010.pdf. All elephant vets, pathologists and caretakers should be acquainted with the protocols in both documents (Elephant Necropsy Protocol and Elephant Endotheliotropic Elephant Herpesvirus (EEHV) Research And Tissue Request Protocol) and should have the necessary equipment ready to facilitate sample collection. A team should be designated in advance for data and sample collection to save valuable time. A list of researchers interested in participating in elephant necropsies is included in the Elephant Necropsy Protocol.

Post-mortem examination of an elephant can be a daunting task, but with proper personnel, planning, and experience, it can be done safely and efficiently. If at all possible, institutions should make preparations or contingency plans for the movement, necropsy, and disposal of an elephant ahead of time to avoid the stress of planning following the death of the animal. The information gained from an elephant necropsy is potentially hugely valuable to institutions, the AZA, and to elephants both in human care and in the wild.

Scott Terrell, DVM, Dipl. ACVP
Head, Department of Pathology
Veterinary Services, Disney's Animal Kingdom
AZA Elephant TAG/SSP Pathology Advisor

1200 N Savannah Circle
Bay Lake, FL 32830
Work: (407) 938-2746 Fax: (407) 938-1909
Home: (407) 251-0545; Cell: (321) 229-9363

Email: Scott.P.Terrell@disney.com

Michele Miller, DVM, PhD
Chief Veterinary Officer
Palm Beach Zoo
AZA Elephant TAG/SSP Veterinary
Advisor
1301 Summit Blvd.
West Palm Beach, FL 33405
Cell: 561-727-9630
Work: 561-833-7130 ext 224
Fax: 561-833-7135

Email: mmiller@palmbeachzoo.org

RESEARCH REQUESTS AND CONTACT INFORMATION

1. Baylor University

Jeff Stanton, DVM or Paul Ling, PhD
Department of Molecular Virology & Microbiology
Baylor College of Medicine
Mail Stop BCM-385
One Baylor Plaza
Houston, TX 77030
Work: (713) 253-9282 (Jeff Stanton)
Email: jstanton@bcm.edu; pling@bcm.edu

Trunk Wash Collection Protocol for EEHV Screens: Generally follow standard trunk wash collection protocol for *M. tuberculosis* culture: Instill 50mL sterile saline solution into the nares. Elevate the trunk 2-3ft for 30-60 seconds. Have elephant blow into a clean container – use new ziplock/plastic bags for each sample to reduce the chance of cross contamination. Transfer sample to a 50mL conical tube – the more mucous and exfoliated respiratory epithelial cells the better (SNOT IS GOOD). Chill the sample on ice until it can be centrifuged. Centrifuge at 1500Xg for 10min @ 4°C or room temperature. Gently pour off and discard the supernatant. Ideally the cell pellet is stored @ -80°C and shipped on dry ice, however it is acceptable to store cell pellets @ -20°C and ship on ice.

2. National Elephant Herpes Lab

Erin Latimer/Laura Richman/Gary Hayward (John Hopkins)
Smithsonian, National Zoological Park
Department of Pathology
3001 Connecticut Ave. NW
Washington, D.C. 20008
Work: (202) 633-4252
Email: latimere@si.edu

1. Serum – 2 mls collected weekly; transfer to plastic screw-top tube and store at -80C or non-defrosting freezer until shipped (for EEHV titers). *Contact Debbie Olsen (dolson@elephantconservation.org) for study number to label tubes (use study number and date collected ONLY). Ship samples quarterly when requested by email, overnight with ice packs or dry ice. **2. Whole blood** – 1-2 mls in EDTA tube, then transfer to plastic screw-cap for storage at -80C freezer until shipped (for EEHV detection). **3. Placenta** – freeze 1 inch³ piece in liquid nitrogen or dry ice, then store at -80C freezer until shipped. Also, serum and whole blood from dam and baby. **4. Herpes lesions** – wet a cotton swab with small amount of sterile saline, swab lesion and place in sterile 15 ml plastic test tube; store at -80C until shipped. **5. Saliva** – scoop up saliva into 15 ml sterile plastic tube; store at -80C until shipped. **6. Necropsy tissues (heart, liver, tongue, spleen, intestine, any tissue with hemorrhages, any kind of nodules – skin, lung, vestibular)** – aseptically place 3x3 cm piece of tissue in small Ziploc or WhirlPak bag. Label with type of tissue, elephant ID, date. Use separate bag for each tissue; store at -80C until shipped. **7. Urine, semen** – place up to 5 ml in a sterile plastic tube (50 ml for trunk wash); store at -80C until shipped. **8. Trunk Wash** – centrifuge or send immediately on ice. **Shipping** – FedEx overnight; email tracking number to latimere@si.edu. EEHV Lab will pay for shipping of sample from healthy elephants; contact lab for account information.

3. Cornell University

Mary Beth Matychak/Noha Abou-Madi/Julia Flaminio
Cornell University, College of Veterinary Medicine
Department of Clinical Sciences
Room C3521
Ithaca, NY 14853
Work: (607) 253-3493
Email: mbm10@cornell.edu

1. Whole blood from EEHV infected or surviving elephants-10-30 mls whole blood in EDTA, ideally collected prior to starting treatment, and every 6-8 hours if possible (if bled again). Samples should be sent overnight on ice for morning delivery (for EEHV culture). **2. Umbilical cord vessels and blood** – notify investigators of birth 1-2 days ahead for shipping supplies (email – mbm10@cornell.edu, na24@cornell.edu). Once placenta passed, mix 10 mls antibiotic solution in each of liters of saline provided. Identify end of umbilical cord closest to calf, cut and discard first 2 inches of cord, then harvest vessels in segments of 1-1.5 feet. Do not tie segments into knots. Use saline bottles to rinse outside of cord; don't rinse inside of cord. Transfer 300 ml of saline into empty one and place section in saline bottles. Wrap tape around neck of bottles; wrap bottle in 1-2 diapers and place in baggie. Place in Styrofoam box, add 2 ice packs. Collect blood from any remaining cord into EDTA tubes. Ship overnight for AM delivery (culture of endothelial cells). **3. Necropsy tissues (heart, lung, liver, spleen, tongue, skeletal muscle, brain)** – collect 2-3 cm² samples and place in individual containers of antiviral transport media (supplied by investigators). Collect pericardial and peritoneal fluid in EDTA tubes. Ideally, collect 10-30 mls EDTA whole blood pre- or immediately post-mortem. Ship samples on ice packs overnight for AM delivery (EEHV culture). **Shipping** –Contact the lab for shipping/FedEx information. Contact Dr. Mary Beth Marychak or Noha Abou-Madi (607-253-3278).

4. Houston Zoo

Joseph Flanagan/Lauren Howard
Houston Zoo
Veterinary Hospital
1513 North MacGregor
Houston, TX 77030
Clinic: (713) 533-6632
Email: jflanagan@houstonzoo.org/lhoward@houstonzoo.org

Serum from confirmed EEHV cases – 10-100 mls serum for EEHV sequencing. Place serum in screw top plastic tubes and store at -80C until shipped on dry ice. Call ahead or email prior to shipping.

5. Cornell University

Jeff Talcott/Noha Abou-Madi
Animal Health Diagnostic Center
College of Veterinary Medicine
Cornell University
Upper Tower Road
Ithaca, NY 14853
Work: (607) 253-3900
Email: na24@cornell.edu

Necropsy tissues (lung nodules) - 0.5 cm by 0.5 cm should be collected from lung nodules (two samples each) and placed in a container with antiviral transport media (supplied by investigator). The sample should be shipped back on ice packs overnight for AM delivery. Contact lab for shipping/FedEx information (EEHV isolation).

6. John Hopkins School of Medicine

Gary Hayward
Johns Hopkins School of Medicine, Viral Oncology Program
3M09, Bunting-Blaustein Cancer Research Building,
1650 Orleans St,
Baltimore, MD 21287 (Fedex)
PH 410-955-8684 Fax 410-955-8685 (work)
PH 410-821-8197 (Home/weekend)
Email: ghayward@jhmi.edu
Contact lab for FedEx information

1. Necropsy of All Asymptomatic Wild-Born Adult Asian and African Elephants: Lung Nodules and EEHV Latency:

- (a) Multiple small pieces of as fresh as possible unfrozen lung tissue from breadloaved bronchiolar area. Transport on ice in 40ml sterile plastic tubes. **Please also collect multiple "palpable" lung nodules if present.** Different nodules from the same animal have proven to contain different EEHV species. Small nodules are white and ovoid with smooth surfaces and 2-3mm in size embedded within the parenchyma. Larger ones may be gray/white and spongy. We have also been able to detect virus at low levels even in nearby non-nodular bronchiolar tissue--perhaps because there are "micro-nodules". Fresh tissue sent on ice packs is preferred. But if tissues have been frozen before transport, do not thaw. Send frozen tissue with ice packs in an insulated Styrofoam container.
- (b) Fresh lung necropsy tissue (several small pieces) from upper airway for epithelial cell culture. Transport unfrozen with ice packs in 40 ml plastic tubes containing PBS plus antibiotics.

2. Fresh Unfrozen Blood and Serum from All Live Confirmed EEHV Viremia Positive Animals: Virus Culture and EEHV Whole Genome DNA Sequencing.

- (a) Blood and Serum: Fresh heart blood (2ml to 4ml) and serum (20ml to 200ml in butterfly catheter: ie **as much serum as possible please**) transported on ice/refrigerated packs. In late stage untreated viremic cases and non-responders a great deal of virus is released as cell-free virions into the serum. This has not been the case in early stage acute disease or in drug-treated survivors. Cell-free virus is needed for attempts at deep sequencing of intact EEHV genomes. Unfrozen blood samples will be used for PBMC fractionation and virus cell culture attempts in primary elephant vascular endothelial cells. **Preferably please collect and ship a first set of samples obtained before or at the same time that FCV/GCV treatment is initiated.** A second sample at 24 or 48 hours (and subsequent ones if desired) after treatment will allow us to evaluate the effectiveness of the medication in terms of increase or decrease of viral load.

3. Necropsy of EEHV-Positive Hemorrhagic Disease Case. Virus Culture and EEHV Whole Genome DNA Sequencing.

- (a) Blood and Serum (as above). **As much serum as possible please----may be frozen in this case or on ice.**
- (b) Fresh unfrozen tissue with hemorrhaging (eg heart, lung, spleen, liver or tongue) for further attempts at so far unsuccessful virus cell culture. Ship directly on ice in 40 ml sterile plastic tubes or with small volume of transport medium (PBS plus Pen/Str/Fungizone or similar)

4. Birth of Asian or African Elephant Calf. For Primary Endothelial Cell, Epithelial Cell and Lymphocyte Cell Cultures. [All samples for culture are shared with Virginia Pearson, Visiting Research Fellow at Princeton University].

- (a) Fresh unfrozen umbilical cord (several 8 to 12 in. segments) in 1x or 2x 500ml wide-mouth bottles provided with sterile PBS plus antibiotics (wash thoroughly with contents of second bottle). Transport on ice/refrigerator packs ASAP with return collect Fedex pre-package that will be provided.
- (b) Fresh unfrozen whole cord blood (4-10 ml if possible) in EDTA tubes for PBMC fractionation. Transport on ice/refrigeration packs provided.

7. Princeton University

Virginia R. Pearson
Guest Researcher, Department of Molecular Biology
Princeton University,
Princeton, NJ 08544, USA
mobile 215-816-5734, FAX 215-247-1287
vpearson@princeton.edu

1) From healthy African and Asian elephants primary cell culture: Birth of Asian or African Elephant Calf. For Primary Endothelial Cell, Epithelial Cell and Lymphocyte Cell Cultures.

(a) Fresh unfrozen umbilical cord (several 8 to 12 in. segments) in 1x or 2x 500ml wide-mouth bottles provided with sterile PBS plus antibiotics (wash thoroughly with contents of second bottle). Transport on ice/refrigerator packs ASAP with return collect Fedex pre-package that will be provided.

(b) Fresh unfrozen whole cord blood (4-10 ml if possible) in EDTA tubes for PBMC fractionation. Transport on ice/refrigeration packs provided.

(c) Amniotic sac (fetal, still or live birth) 3x 3sq" pieces and 6" section of umbilical cord connecting to placenta - include blood vessels lying over and 1" sq piece of placenta

2) The following necropsy tissues for epithelial cell culture and virus latency investigation. All FRESH NOT FROZEN tissues must be harvested as soon as possible after death and shipped within 24 hours. From sudden death or suspected cases of EEHV for viral latency and transmission study:

1. Aorta - 2 x 6-inch pieces to fit 50ml tube, include section where aorta connects to heart -

2. Heart - 4 x 1sq" pieces from outside wall and inside cavity

3. Trachea and connecting bronchial tubes - cut up to 6 inches to fit in 50ml tubes

2. Bone marrow - fill 50ml tube

3. Salivary gland - intact

4. Trigeminal ganglion - 6" section toward top of trunk if possible.

NOTIFY before shipment and as early as possible about pending euthanasia, expected or sudden deaths to receive shipping reagents. Send invoice for reimbursement of shipping costs. WASH all tissues gently with sterile PBS unless otherwise noted, DO NOT WASH inside of blood vessels; SUBMERGE all tissues in fresh sterile PBS containing Pen/Strep/Fungizone; USE 50ml tubes; KEEP on wet ice until packed for shipping. SHIP on wet ice (DO NOT FREEZE TISSUES) overnight for next morning delivery to:

Virginia Pearson,
701 West Gravers Lane
Philadelphia, PA 19118 .
mobile 215-816-5734



LOGISTICS AND NECROPSY TIPS

The knowledge we have gained and will continue to gain from the elephants held in North America is highly significant for the protection of elephant populations worldwide. There is still much that needs to be done to enable us to be able to prevent and treat this deadly disease in elephants.

The necropsy of an elephant should proceed in the same manner as the necropsy of any smaller mammalian species. Although the size and scope of an elephant necropsy may seem intimidating, the procedure can be accomplished in 8-10 hours (sometimes less) by a team of dedicated prosectors and assistants. The necropsy should be performed with the elephant in left lateral recumbency. An external examination is performed to evaluate body condition and lesions. The oral cavity should be closely examined for evidence of lesions consistent with endotheliotropic herpes virus infection.

Assigning specific tasks to team members will help the necropsy proceed in an orderly manner. For example, a team may be assigned to each of these areas: head, forelegs, hind legs, abdominal region. One person should oversee the collection, labeling, and processing of research materials and any communication concerning research requests. It may be helpful to designate a media spokesperson. One of the most important tasks to be assigned is the task of knife sharpener. One person with knife sharpening experience should be assigned to be continually sharpening knives and cycling sharpened knives to prosectors.

Whole blood and serum samples from sick or dead elephants should be obtained for diagnostic testing in any suspected case of herpesvirus infection.

Small numbers of white to gray nodules with a spongy texture (3-30 mm in cross sectional diameter) in lungs have been found in a high fraction of African elephants culled in the wild and the few that have been examined so far proved to contain high levels of EEHV2, EEHV3 and/or EEHV6 (subclinical or latent infection). These lung nodules have also been reported in Asian elephants and a thorough search for lung nodules by slicing through the lung at regular intervals (“breadloafing”) with palpation at necropsy should facilitate collection of such nodules in both Asian and African elephants. The nodules may be very small and rare within the lung, or could be obvious and more numerous and are now expected to be found in most otherwise healthy elephants. In the absence of obviously visible nodules we are also requesting random lower bronchiolar tissue samples.

Similarly, raised skin nodules with darker fibrous centers have been found occasionally in otherwise healthy juvenile African elephants and in one outbreak in Florida; these contained EEHV1. A third type of lesion has been associated with EEHV1: variably sized, red ulcers or vesicles in the distal vestibulum of the genital tract of African elephants. More samples of all of these types of lesions (lung and skin nodules, vestibular ulcers/vesicles) are required from both captive and wild Asian and African elephants to evaluate the natural history of the EEHVs. Please search carefully for and collect “benign herpes” lung nodules especially in all elephant necropsies.

ELEPHANT NECROPSY PROTOCOL GROSS EXAMINATION WORKSHEET

Institution/Owner _____

Address _____

Species _____ ISIS# _____ Studbook# _____

Name _____

Birth date/Age _____ Sex _____ Weight (Kg) _____
Actual Estimate

Death date _____ Death location _____

Necropsy date _____ Necropsy location _____

Post mortem interval _____

Captive Born Wild Caught

History (clinical signs, circumstances of death, clinical lab work, diet & housing)

GROSS EXAMINATION

(If no abnormalities are noted, mark as normal or not examined (NE); use additional sheets if needed)

General Exam (physical and nutritional condition, skin, body orifices, superficial lymph nodes). **Skin nodules have been associated with EEHV in African elephants* (samples for fresh/frozen/formalin should be saved).**

Musculoskeletal System (bones, marrow, joints, muscles)

Body Cavities (fat stores, pleura, thymus, lymph nodes)

Spleen

Respiratory System (trunk passages, pharynx, larynx, trachea, bronchi, lungs, regional lymph nodes; submit lung lesions for TB culture. **Bronchial lymph nodes should be cultured for TB even if normal in appearance**). **Lymphoid nodules in lungs may be associated with EEHV infections*** (samples for fresh/frozen/formalin should be saved).

Cardiovascular System (heart, pericardial sac, great vessels, myocardium, valves, chambers) **Be sure to closely examine abdominal aorta for subtle or obvious aneurysms**

Digestive System (mouth, teeth, tongue, esophagus, stomach, small intestine, cecum, large intestine, rectum, liver, pancreas, mesenteric lymph nodes)

Urinary System (kidneys, ureters, bladder, urethra)

Reproductive System (testes/ovaries, uterus & cervix, penis/vagina, urogenital canal, prostate, seminal vesicles, bulbo-urethral gland, mammary gland, placenta). **Uterine masses/tumors are extremely common in Asian elephants and multiple tumor types may be present.**

Endocrine System (thyroids, parathyroids, adrenals, pituitary)

Central Nervous System (brain, meninges, spinal cord)

Sensory Organs (eyes, ears)

Additional Comments or Observations:

Prosector: _____ Date: _____

Summarize Preliminary Diagnoses:

Laboratory Studies: Please attach results of cytology, fluid analysis, urinalysis, serum chemistries, bacteriology, mycology, virology, parasitology, x-ray, photographs, or other data collected.

TISSUE CHECKLIST

Freeze 3-5 cm blocks of tissue from lesions and major organs (e.g., lung, liver, kidney, spleen) in small plastic bags. Freezing at -70 degrees Celsius in an ultra-low freezer is preferred. If this is unavailable, freezing at conventional temperatures is acceptable (use a freezer without an automatic defrost cycle if possible).

Any lesions noted in the lungs should be submitted to NVSL or other qualified mycobacterial laboratory for mycobacterial culture (ie. National Jewish Diagnostic Lab, Colorado). Bronchial lymph nodes should be cultured for TB even if normal in appearance. Preserve as many of the tissues listed below as possible in 10% buffered formalin at a ratio of approximately 1 part tissue to 10 parts solution. Tissues should be no thicker than 0.5 to 1.0 cm. Fix diced (1x1 mm) pieces of kidney, liver, spleen and lung in a suitable EM fixative if possible - glutaraldehyde base e.g., Trump-McDowell fixative. NOTE: There is generally no need to fix and label each tissue separately. Take 2 sets of fixed tissue. Bank one set. Send tissues required for diagnosis to primary pathologist and request a duplicate set of slides for the SSP pathologist, Dr. Scott Terrell who should be contacted for further instructions. Also, freeze post mortem serum (from heart), urine and any abnormal fluid accumulations. Consult **Elephant Research and Tissue Request Protocol** for specific project sample requests. Consult for specific sample requests, which sometimes includes fresh chilled but unfrozen tissue from necropsy for cell culture, Johns Hopkins and Princeton University.

- | | | | |
|---|---|--|---|
| <input type="checkbox"/> Adrenal | <input type="checkbox"/> Kidney | <input type="checkbox"/> Penis | <input type="checkbox"/> Thymus |
| <input type="checkbox"/> Blood * | <input type="checkbox"/> Large intestine | <input type="checkbox"/> Pituitary | <input type="checkbox"/> Tongue |
| <input type="checkbox"/> Bone with marrow | <input type="checkbox"/> Liver | <input type="checkbox"/> Prostate | <input type="checkbox"/> Trachea |
| <input type="checkbox"/> Bulbo-urethral gland | <input type="checkbox"/> Lung | <input type="checkbox"/> Salivary gland | <input type="checkbox"/> Trunk cross section |
| <input type="checkbox"/> Brain | <input type="checkbox"/> Parathyroid | <input type="checkbox"/> Temporal gland | <input type="checkbox"/> Seminal vesicles |
| <input type="checkbox"/> Cecum | <input type="checkbox"/> Mammary gland | <input type="checkbox"/> Skin | <input type="checkbox"/> Ureter |
| <input type="checkbox"/> Diaphragm | <input type="checkbox"/> Muscle | <input type="checkbox"/> Small intestine | <input type="checkbox"/> Urinary bladder |
| <input type="checkbox"/> Esophagus | <input type="checkbox"/> Nerve (sciatic) | <input type="checkbox"/> Spinal cord | <input type="checkbox"/> Vaginal/urogenital canal |
| <input type="checkbox"/> Eye | <input type="checkbox"/> Ovary/testis | <input type="checkbox"/> Spleen | <input type="checkbox"/> Uterus/cervix |
| <input type="checkbox"/> Hepatic bile duct | <input type="checkbox"/> Epididymus | <input type="checkbox"/> Tonsillar lymphoid tissue | |
| <input type="checkbox"/> Heart/aorta | <input type="checkbox"/> Pancreas | <input type="checkbox"/> Stomach | <input type="checkbox"/> Thyroid gland |
| <input type="checkbox"/> Hemal node | <input type="checkbox"/> Lymph nodes (tracheobronchial, submandibular, tonsillar, mesenteric) | | |

* Collect post mortem blood, separate serum and freeze for retrospective studies.

Primary Pathologist (Name): _____

Lab

Address _____

Phone _____

(Please send a copy of this protocol with gross descriptions and preliminary diagnoses to SSP pathologist and SSP veterinary advisor. Send final report with histopathologic findings, laboratory results, and any pertinent digital or color slides) to (and copy to SSP veterinary advisor):

Scott P. Terrell, DVM, Diplomate ACVP

SSP Pathology Advisor, Elephants

Disney's Animal Kingdom, 1200 N Savannah Circle, Bay Lake, FL 32830

W (407) 938-2746; H (407)251-0545; Cell (321)229-9363mail: Scott.P.Terrell@disney.com

Michele Miller, DVM, MS, PhD

SSP Veterinary Advisory

mmiller@palmbeachzoo.org

CONSENT FORM FOR USE OF SAMPLES BY AZA ELEPHANT TAG/SSP

I give consent for the sample submitted to the AZA Elephant TAG/SSP serum/tissue bank to be used for research on any elephant related issues based on recommendations by the veterinary advisor and/or steering committee.

The results could be reviewed and used by the AZA Elephant TAG/SSP Veterinary Advisor in providing health-related recommendations and publications.

I understand that all results and recommendations regarding the individual elephant will be kept confidential.

_____ Yes, I agree to allow the AZA Elephant TAG/SSP to use our sample for designated research and testing results.

_____ No, I do not consent to the use of our sample and test results unless specified.

Signature, title

Date

Printed name

Phone number

Institution

Email address

Address

Comments: _____

FREQUENTLY ASKED QUESTIONS ABOUT EEHV

In the wild, elephants face extreme pressure from human-elephant conflict, habitat loss and poaching. In North America, elephants are important conservation ambassadors for their species and ecosystems. Seeing, hearing, and even smelling these magnificent animals up close is critical to helping visitors make an emotional connection to the natural world of elephants and take action to help protect their future. We need elephants in human care if we are to save them.

There are many questions about this complex group of viruses. We hope these questions and answers help you better understand as well as explain to others these viruses and the diseases they can cause.

What do we know about elephant herpesviruses?

To date, scientists have identified 14 genetically different elephant herpesvirus types, five of which are known to cause hemorrhagic disease. The viruses found in symptomatic elephants at different zoos and other institutions are genetically distinct, which means that they are not all the same strain spread by the transfers of elephants between and among zoos.

Herpesviruses are widespread in all mammal species, including humans. While species-specific, they share common features. Once inside a host, the virus can go into a latent (hidden) phase after causing only mild symptoms or no signs of disease at all. Scientists do not yet know where in the body EEHV resides in the latent phase.

For reasons unknown, primary or reactivated latent elephant herpesvirus infections can sometimes circulate uncontrolled throughout the bloodstream, causing disease. This is the only time when a herpesvirus can be readily detected in blood samples. As yet, reliable tests are not available to detect a latent (hidden) infection. Most elephants are able to fight the virus and survive when it comes out of latency. Calves appear to be most susceptible to EEHV disease after they have been weaned, at a time when they are not protected by their mother's antibodies.

Does EEHV affect elephants only in zoos?

We know that EEHV is not just a disease of the captive Asian elephant in western countries. According to an International Elephant Foundation progress report of spring 2009 by EEHV experts, more than a dozen cases of EEHV have been identified in elephant populations in India, Thailand and Cambodia – including several wild as well as orphaned Asian elephant calves that have died within the past few years. Moreover, these deaths only represent the cases in which necropsies were conducted in sufficient time to detect it.

Current research indicates that the elephant-specific herpesvirus may have been in elephant populations for tens of millions of years, just as human herpesviruses have been in human populations. Since this is a naturally occurring disease, every elephant – in the wild and in human care – probably carries one or more forms of elephant herpesvirus within them.

If elephants in both zoo and wild populations probably have one or more herpesvirus, why do some get ill and others don't?

Many animals and humans carry herpesviruses throughout their lives and never become ill. What researchers don't know is what triggers the virus to become active and where exactly in the body the virus

hides in its latent phase. We don't know why some animals become ill and others don't. It's important to understand that it's not about *who has* the virus, but *who gets ill* and *when*.

Can elephants transmit EEHV to other elephants?

There is not enough research to confirm how EEHV itself is transmitted, but that is normally how viruses spread. Viral shedding occurs when it comes out of latency and most human herpesviruses are transmitted predominantly in saliva. Until recently EEHV could only be detected when active through a blood test, but new studies now suggest that some healthy Asian elephants periodically shed low levels of EEHV1 in secretions from the trunk (which may or may not be infectious). What we do know is that many elephants – in the wild and in human care – probably carry one or more forms of latent herpesvirus within them.

Can the elephant herpesvirus be transmitted through semen?

- There is no evidence of shedding of virus into semen or transmission of EEHV through natural breeding or artificial insemination.
- **There is no evidence to suggest that EEHV is being transmitted between elephants through transport and breeding activities. At present no two facilities have been found to have disease caused by the same strain of EEHV1, they are all different. Therefore, the AZA Elephant TAG/SSP recommends that institutions continue to exchange elephants and elephant semen for breeding and artificial insemination as specified in the breeding recommendations.**

Is a facility contaminated once an outbreak of EEHV has occurred?

Like all mammals and humans, elephants carry a variety of different herpesviruses throughout their lives. Some cause mild disease and some cause severe disease or death. This is how herpesviruses operate. Claims that certain zoos are contaminated once an animal becomes ill from EEHV are unfounded and based on a lack of understanding of how the viruses live within their hosts. Having a herpesvirus is the norm, not the exception. Like all viruses, herpesviruses cannot live very long outside the body, so a herpes outbreak does not “contaminate” a facility.

Is there a cure for EEHV?

There is no cure for herpesviruses in animals *or* in humans. Based on what we are learning from our ongoing research and from elephant care institutions that have experienced an EEHV outbreak, the treatment protocols continue to improve, and detection and treatment recommendations continue to evolve.

Current treatments suppress EEHV and elephants can potentially recover if treatment starts early. Of the elephants that have been treated, the success rate with anti-viral therapy against EEHV has been about 40 percent. Veterinarians and scientists continue to collaborate to better understand this disease and develop more effective treatment options. To date, anti-viral drugs have been used successfully in treating eight Asian elephants in North America.

Shouldn't zoos discontinue breeding elephants if calves are at risk for EEHV?

Stopping breeding in zoos will severely impede the progress that is being made in studying EEHV and finding a cure. Running away from captive breeding is not the way to solve the disease. When an outbreak of equine herpes occurred in 2005 in horses, the industry did not shut down. Instead it funded

research that resulted in treatment, prevention and control of that disease. When black-footed ferrets were nearly driven to extinction in the 1980s from canine distemper, we did not stop breeding them. U.S. Fish and Wildlife Service, AZA institutions, private landowners, conservation organizations, and other groups collaborated on a rescue and recovery program. An effective vaccine was developed and the species bounced back from the brink of extinction.

But why take the risk of exposing another calf to EEHV?

While we have no guarantees as to the fate of a future elephant calf, we have operated for many years under the conservative assumption that all elephants could have one or more latent (hidden) herpesviruses. The risk is no higher or lower for an elephant born in the wild or at a zoo or sanctuary. We will continue to gather the evolving research and use the latest information to guide our decisions in caring for elephants.

Is further research being done to learn more about EEHV?

Multiple research teams worldwide are dedicated to investigating this set of diseases, to understanding how to protect elephants in human care and in the wild, and to solving the mystery of how EEHV is spread and developing an effective vaccine for the virus.

The collaborative work to better understand EEHV may have important implications for wild elephants in the future. Wildlife biologists may one day need to draw upon the growing body of work and knowledge generated by the international elephant community to contribute to the long-term survival of the species both wild populations and those in human care.

