INTERIM REPORT
EEHV Prevalence in Semen Samples of Asian and African Elephants

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2. List the overall and specific conservation needs this project addressed.

This project will determine the prevalence of Elephant Endotheliotropic Herpesvirus (EEHV) in elephant semen of Asian elephants. Asian elephants are endangered and listed under Appendix I of the Convention of International Trade of Endangered Species and Wild Fauna. Today, one of the greatest threats to their survival is EEHV, which has an 80% mortality rate. EEHV is the leading cause of death in elephant calves in North American captive populations, and it is also present in wild populations of elephants [Zachariah, 2013]. It is characterized by swelling of the head with fluid (edema), a purple color of the tongue (cyanosis), lethargy, anorexia, and severe hemorrhages of capillaries in the skin; it can kill an elephant within 4-7 days after symptoms first appear [Richman, 2000]. However, EEHV can also latently infect an elephant, in which the elephant only carries the virus quiescently and does not show symptoms [Latimer, 2011]. The transmission of EEHV is still unclear, so this project aims to determine if EEHV can be transmitted through semen, which has important implications for natural breeding and artificial insemination (AI). Because of the endangered status of the Asian elephant and the restrictions placed on trade of this species, breeding of this animal is becoming more and more dependent on artificial insemination. Recently a genome resource bank (GRB), which stores semen for future use, has been developed for Asian elephants due to improvements in cryopreservation [Kiso et al, 2012]. The GRB provides a "genetic insurance policy" for elephants by preserving genetic diversity in this endangered species. It also can store genetic material from wild bulls that would not otherwise be involved in breeding in captive herds. We will determine if EEHV is present in semen, and, if so, whether the virus can cross-contaminate other semen samples when stored for artificial insemination. The goal of this project is to determine the prevalence of EEHV in frozen raw semen and seminal plasma samples of Asian elephants in North America. A limited number of semen/seminal plasma samples from African elephants will also be tested.

3. Summarize the goals and objectives and describe any changes in goals and objectives from the original proposal.

The goals of this project are two-fold:

1. Test Asian and African elephant semen samples for PCR inhibitors.
   PCR inhibitors are any sort of material in the sample that would prevent PCR reactions from amplifying DNA normally. These inhibitors can be a naturally occurring component of the sample or could be an environmental contaminant in the sample (trunk wash samples of elephants often have PCR inhibitors present, due to the presence of soil, dust, and food in the trunk). PCR inhibitors in a sample would cause false negative reactions (a sample would seem to be negative for EEHV, but in actuality, the sample could have EEHV present that couldn't be amplified because of the inhibitors). PCR can be negatively affected if PCR inhibitors are present in the sample, which might prevent amplification of the DNA by interfering with the polymerase, by sequestering some of the needed cofactors of the reaction, or by interacting with the DNA. Before screening the samples for the presence of EEHV, it will be verified that the DNA samples are free of PCR inhibitors.

2. Determine the prevalence of EEHV in the samples.

The prevalence of EEHV in the samples will be determined by conventional (cPCR) and real-time quantitative (qPCR) polymerase chain reaction.

4. For each objective, describe the specific actions taken to achieve that objective.

To test the samples for PCR inhibitors, conventional and quantitative PCR are used in order to amplify an elephant gene from the samples. Because the samples contain abundant amounts of elephant DNA, elephant genes should be amplifiable and detectable after PCR. Primers directed to elephant genes ((Tumor Necrosis Factor (TNF) and/or gamma interferon (g-IFN)) are used to amplify the elephant genomic DNA. If the amplified DNA is detected, PCR inhibitors are either not present in the sample or present in low enough quantities that they do not impede PCR.

One hundred thirty five samples (mostly seminal plasma samples) have been received by the National Elephant Herpes laboratory (NEHL) for testing. These samples have been collected by Dr. Wendy Kiso as part of her research on the cryopreservation of elephant semen. Additional samples may be collected by Dr Wendy Kiso and shipped to the NEHL as available.

DNA has been prepared from 73 of the 135 samples. In our initial testing for PCR inhibitors, thirteen of 73 (18%) samples did not produce the internal positive control using PCR. Samples that are not initially positive for the internal positive control will be treated by whole genome amplification to dilute out the PCR inhibitors and then re-tested for the internal positive control.

To screen for EEHV in Asian elephant samples, primers specific to EEHV1, EEHV3/4, and EEHV5 (the EEHV types which have been found in Asian elephant blood, tissues, and trunk wash samples) are used to amplify EEHV DNA through cPCR. To screen for EEHV in African elephant samples, primers specific to EEHV2, EEHV3/4, and EEHV6 (the EEHV types which have been found in African elephant blood, tissues, and trunk wash samples) are used to amplify EEHV DNA through cPCR. Additionally, a primer set that detects all of the known EEHV viruses is used as an additional detection method on both Asian and African elephant samples. Once the DNA has been amplified, it is run through gel electrophoresis to be identified visually by band size. If a band corresponding to the relevant gene is visualized, it is cut out, purified, and sequenced. The sequence of the band is then compared to known sequences of EEHV DNA to verify that the amplified DNA is from EEHV.

The EEHV-specific PCR is in progress on the first set of 73 DNA preparations.

5. Describe any activities that differ from the original proposed actions and explain the reason for the change.

The primers used for performing cPCR for the gIFN gene were of variable quality and the TNF primers had some cross-reactivity with PCR components, so qPCR is now being used for the internal positive control testing.

6. Describe the conservation outcomes for elephants, wildlife, habitat and human communities, and list major findings and accomplishments to date.

Elephants are beautiful and majestic animals that are revered around the world every day in zoos and in the wild. Today, the Asian elephant is listed as endangered and the African elephant as vulnerable, so people can no longer remove them from wild habitats. Within 50 years, it is estimated that current zoo populations of elephants will be extinct because of high mortality rates and low breeding success in populations in human care, as estimated by current conception and attrition rates [Fickel 2000]. This startling statistic helps to explain why elephant breeding is so dependent on safe artificial insemination (AI) techniques and why research on EEHV, a deadly elephant disease, is so important. It is critical to know whether EEHV is present in and can be transmitted by semen to ensure the safety of AI and improve the storage methods of semen that is used for AI. Additionally, AI may be used as a way to incorporate genetic material from wild bulls into captive populations to maintain genetic diversity. Because EEHV is also found in wild populations, it is of particular importance to know if the virus is present in semen, especially if the history of prior infection or exposure to EEHV in the wild bull is unknown [Zachariah, 2013].

Wendy Kiso has provided 135 samples for testing. 73 samples have been tested for PCR inhibitors, and all but 13 samples did not contain PCR inhibitors. EEHV testing on those 73 samples is in progress.

7. Describe any problems discovered or occurring during this grant period.

The TNF primers were cross-reacting with PCR components, so we are now doing qPCR for the internal positive control testing. qPCR uses a TNF-specific probe with the primers, which increases the specificity of the assay, so the cross-reactivity is not problem.

8. Was your project successful? State short and long term goals that you are using to evaluate your accomplishments.

DNA has been prepared from approximately half of the samples received so far. Less than 20% have had evidence of PCR inhibitors; thus, most of the samples can be tested for EEHV without further treatment by whole genome amplification.

9. Based on this Project, what is the "next step" for this project and does it have implications for future conservation actions?

We will continue to process semen samples from elephant bulls. A larger sample size will be more useful for determining if EEHV is present in elephant semen. If we find evidence for EEHV in elephant semen, the next step would be to determine if cross-contamination between semen samples is possible in liquid nitrogen storage, the common way of storing semen samples for artificial insemination.

10. Provide at least one human interest story. This story should enable the reader to identify with the people, a problem, day-to-day situations, achievements or a funny or strange occurrence during the course of the project.

Also known as the semen lady, Nicole Furst is the intern at the NEHL during the summer of 2015. In the lab, Nicole will determine the prevalence of EEHV in elephant semen to learn more about the transmission of this disease and how this would affect samples for artificial insemination. The unenviable job of collecting the elephant semen falls to Dr. Wendy Kiso. Nicole extracts DNA from the semen, which contains a large amount of elephant DNA and possibly EEHV DNA. Then, she amplifies the DNA with PCR, views it using gel electrophoresis, and sequences any possible EEHV DNA. Though this whole process may just sound like basic biology, pipetting semen and micro amounts of DNA all day is profoundly important for Nicole and ultimately for elephants. Nicole is involved in the huge effort to save endangered elephants from extinction by preventing elephant deaths due to EEHV. For this soon to be veterinary student, this is an important first step towards becoming a zoo and wildlife veterinarian. While working in the lab, Nicole is learning about the epidemiology of diseases that affect zoo animals and wildlife. The research in the EEHV lab addresses captive and wild elephants all over the world. This internship will help Nicole achieve her goal of becoming a zoo animal veterinarian and conducting important research that has a global impact. For these reasons, Nicole looks forward to every day of her internship especially because she knows the ultimate goal of her work is to improve the lives and health of elephants around the world.

11. In 500 words or less, summarize the progress and results achieved. this will be used for media and donor recruitment.

The Smithsonian's National Zoo, Ringling Bros. Center for Elephant Conservation and the International Elephant Foundation have teamed up to determine the prevalence of EEHV in elephant semen. In this study, we are testing approximately 150 semen and seminal fluid samples from mostly Asian and some African elephants. We wish to determine if EEHV is present in semen samples from elephants. Although there has been no evidence of EEHV transmission through natural or assisted reproduction, it is not known if EEHV is present in the semen of Asian or African elephants. This will be the first systematic study to determine the prevalence of EEHV in semen.

We will test approximately 150 samples from up to 20 mostly Asian bulls. Approximately half of the samples have been processed to prepare the DNA. Most samples (about 80%) do not contain PCR inhibitors, so they will be screened for EEHV without further processing. We will screen the samples for EEHV 1, 2, 3-4, 5 and 6 by polymerase chain reaction (PCR). We have tested about 15% of the samples and thus far, we have found no evidence for EEHV in our samples.

If we do find evidence that EEHV is shed in semen samples, the next step will be to develop screening and storage methods to ensure that EEHV-free semen is used in artificial insemination. We want to ensure the future of these charismatic and magnificent animals.

12. In 50 words or less, summarize the progress and the results achieved. This will be used for social media.

The Smithsonian's National Zoo, Ringling Bros. Center for Elephant Conservation and the International Elephant Foundation have teamed up to determine the prevalence of EEHV in elephant semen. So far, we have found no evidence for elephant endotheliotropic herpesvirus

(EEHV) in fifty Asian elephant bull semen samples after testing for three different types of EEHV. We plan on testing up to 150 samples from about 20 bulls.

13. List all organizations associated with this project and their roles in the project.

International Elephant Foundation (IEF), funding for supplies
Ringling Bros. and Barnum & Bailey, funding for intern stipend
Ringling Bros. and Barnum & Bailey Center for Elephant Conservation (CEC), Wendy Kiso & Dennis Schmitt, co-investigators
National Zoological Park (NZP), Pierre Comizzoli,Erin Latimer, co-investigators; Nicole Furst, intern

14. Include a financial report of International Elephant Foundation funds spent.

budget item	*allocated*	*spent*	*remaining*
DNEasy Blood kit	$665	$665	$0
Repli-g kit	$1,224	$612	$612
FedEx shipping	$480	$20	$460
Qiaquick kit	$514	$0	$514
cPCR	$6,000	$4,192	$1,808
Total	$8,883	$5,489	$3,394

15. Submit at least 5 pictures.
Captions for pictures:

1. 00986—Dr. Wendy Kiso is shown with a liquid nitrogen tank. Liquid nitrogen is used to freeze samples at −195.79 °C (−320 °F).
2. 02846—Dr. Wendy Kiso is shown with an elephant friend.
3. 464—Intern Nicole Furst is shown at the biological safety cabinet, preparing DNA from semen samples.
4. 469—Nicole pipetting ul quantities of liquid during a DNA prep.
5. 521—Nicole loading an agarose gel, which is used to separate DNA by size.
6. qPCR—graph showing the internal positive control qPCR assay. This shows that the DNA preparations are of good quality and don't have PCR inhibitors. The positive control is an elephant gene, TNF.

16. Submit at least one video clip.

17. A copy of all future publications that result from this study.

A paper will be written for submission to Journal of Andrology or Theriogenology; EL will present the results at the annual EEHV Workshop. A story about the study will be featured in the NEHL Report.

18. Please list all websites, blogs, social media accounts, etc associated with the project, its investigators, and organizations.

http://www.eehvinfo.org/

http://nationalzoo.si.edu/scbi/animalcare/eehv/

Literature Cited

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Kiso, WK, Asano, A, Travis, AJ, Schmitt, DL, Brown, JL, Pukazhenthi, BS. Pretreatment of Asian elephant (Elephas maximus) spermatozoa with cholesterol-loaded cyclodextrins and glycerol addition at 4°C improves cryosurvival. Reprod., Fertil. and Dev. 2012. 24:1134-1142.

Latimer E, Zong JC, Heaggans S, Richman LK, Hayward GS. Detection and evaluation of novel herpesviruses in routine and pathological samples from Asian and African elephants: Identification of two new probosciviruses (EEHV5 and EEHV6) and two new gammaherpesviruses (EGHV3B and EGHV5). Vet Micro. 2011. 147(1-2): 28-41.

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Zachariah A, Zong J, Long SY, Latimer E, Heaggans SY, Richman L, and Hayward GS. 2013. Fatal Herpesvirus (EEHV) Hemorrhagic Disease in Wild and Orphan Asian Elephants in India. J Wild Dis. 49(2):381-93.