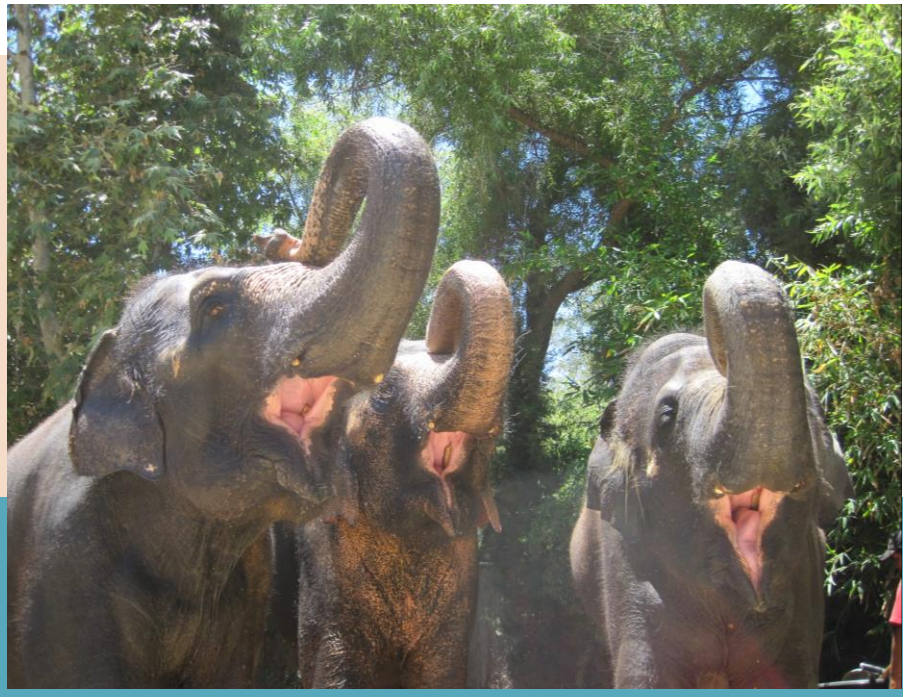
SAYING "AHH"

SNIFFING ELEPHANT BREATH IS A VERY COOL PART OF THE DAY AT HAVE TRUNK WILL TRAVEL.

Every day, they check their teeth and tusks, look for any abnormalities, make sure they see a healthy pink color and even smell the elephant's breath to be sure there is no odor that might indicate an infection or an accumulation of gunk between their teeth. Dixie, Tai and Rosie are obviously used to the routine.

PHOTO BY KARI JOHNSON, HTWT



THE EEHV CONSORTIUM
PO BOX 37012, MRC 5508,
WASHINGTON, DC 20013-7012
NEHL at the National Zoo
2015. Vol 2 #1

The EEHV Consortium Update

at National Elephant Herpesvirus Laboratory

PROFILE:

Dr. Gary Hayward, Johns Hopkins University

(1) HOW DID YOU GET INVOLVED IN EEHV RESEARCH?

I first developed an interest in viruses and DNA as a teenager, which led me to experiment with agarose gel electrophoresis for separating bacteriophage DNA molecules of different sizes for my PhD thesis research. I published the first restriction cleavage patterns of human herpes simplex virus genomes in 1975. During a more than 45 year career in DNA research focused mostly on the molecular biology of the many different types of human herpesviruses, I always also had an interest in conservation issues as well as virus evolution and published the complete genome sequence of chimpanzee cytomegalovirus in 2004. Naturally then when Laura Richman first told me that she thought Kumari had died of a previously unknown herpesvirus infection I jumped at the chance to study the problem, including inviting her to come and do her PhD studies on EEHV in my laboratory at Johns Hopkins.

(2) WHAT ARE YOUR ONGOING RESEARCH PROJECTS?

Together with our close collaborators in Paul Ling's group in Houston we have just assembled the complete 200,000-bp complete genome sequence of EEHV4, the fourth type of Asian elephant Proboscivirus and the first of the GC-rich branch to be so characterized. There has also been major progress in characterizing the most divergent genes from numerous distinct EEHV1 strains directly from pathological samples collected by a concerned consortium of veterinarians and our other collaborators from all around the world. We also keep hoping to get a breakthrough in our attempts to culture and propagate these viruses from clinical samples in laboratory cell culture, and we continue efforts to generate multiple clones for expressing enough antigens from each of the sequenced EEHV species in yeast to develop a robust multiplex serology chip assay that may finally overcome the major problems of antibody cross-reactions between them.

(3) BIGGEST CHALLENGE FOR EEHV RESEARCH?

Understanding why 20% of Asian elephant calves worldwide are susceptible to life-threatening acute hemorrhagic disease when undergoing primary infection with EEHV1 (or sometimes other EEHV types), whereas African elephants which harbor just as many types of EEHV species of their own hardly ever get disease. Other than our very successful virus hunting and diagnostic DNA tests that have identified clinical samples suitable for extensive genetic characterization there is little hope of significant further progress in understanding or combating this devastating disease without a major influx of new research funding. Whilst I still ran an active well-funded research …

CONTINUED ON PAGE 2

USEFUL LINKS FOR EEHV PREPARATION

PASSWORD:
REQUEST FROM
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- EEHV PLANNING PROTOCOLS
- EEHV TESTING LABORATORIES
- PROCEEDINGS OF THE FEB 2015 EEHV WORKSHOP.

EEHV Monitoring and Diagnostic Testing of "At Risk" Juvenile Elephants

Routine monitoring of elephant calves for Elephant Endotheliotropic Herpesvirus (EEHV) by quantitative PCR (qPCR) is proven to detect low levels of EEHV in the blood before clinical signs occur, allowing increased monitoring and early therapeutic intervention if viral levels increase (Stanton et al., 2013). The increased sensitivity of qPCR and multiple rounds of cPCR and the ability to quantify whole blood viral levels with qPCR allows for better management of calves with regard to possible EEHV Hemorrhagic Disease (EEHV HD) development. If qPCR isn't available, multiple rounds of cPCR can be a sufficient, but not ideal, replacement. It is now possible to detect and quantify low levels of EEHV in the blood to distinguish between a calf's subclinical or non-hemorrhagic herpes infection and the much more serious EEHV HD and monitor closely for rapid increases in viral levels. Elephants can have low levels of EEHV in the blood with no or minimal clinical signs (*Stanton et al., 2013*) for up to two months, but possibly for as long as one year.

Trunk wash or saliva screening can also detect shedding of virus (as DNA by PCR) for several months during convalescence after primary viremic infection or occasionally from reactivation of a latent infection. While there may be some overlap between high levels of viremia and shedding, viremia is the only parameter that correlates most consistently with disease. High levels of EEHV in blood are typically found in cases of EEVH HD. Screening trunk wash samples for 2-3 months may allow the determination of the types of EEHV present in the herd, with the caveat that only EEHVs that are being shed in the trunk secretions during the collection period would be detected.

Standard monitoring and testing protocols for elephant owning institutions have been developed, to maximize the knowledge gained by appropriate testing while optimizing the use of resources (time and reagents). Current recommendations include weekly monitoring of whole blood from calves 1-8 years of age and annual trunk wash screening of herds. More details on monitoring can be found at eehvinfo.org. The password is E3HVGroup.

Dr. Gary Hayward

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… laboratory studying human herpesvirus disease it was easy to borrow expertise and a little bit of time and effort from my postdoctoral fellows to clone and express EEHV genes, or carry out IFA and IHC assays or develop specific rabbit antibodies for example, but that is no longer the case, and the number of hands-on laboratory personnel involved have now dwindled down from five or six to just three and dropping. Furthermore, both Virginia Pearson (our collaborator on African elephant herpesvirus identification) and I have been working extensively essentially as unpaid volunteers for several years now. The International Elephant Foundation, the Morris Animal Foundation and a Collections Stewardship Leadership grant from the IMLS have contributed essential funding that has kept our EEHV research projects limping along, but my group will have to totally close down within a year or so without the kind of generous funding from a private donor that currently supports the EEHV research in Paul Ling's laboratory at Baylor College of Medicine in Houston.

(4) Where do you see EEHV prevention, diagnosis and treatment in five years?

No real change. There is at present virtually nothing known about the immunology of the elephant hosts themselves or of this novel new group of mammalian herpesviruses. Without cell culture and lots of funding, there is also really no realistic hope of vaccines within the near future, and it is only the ability to carry out close blood test DNA monitoring of calves and to respond to "viremic" illness with rapid good medical care that has improved the management of the disease in the USA in recent years. But it has been a major financial challenge just to keep the dedicated expertise necessary for this in Erin Latimer's NEHL diagnostic laboratory, as well as in my molecular genetic strain subtyping group, together from one year to the next, let alone get serious about any more extensive efforts at finding better anti-EEHV drugs or exploring the mechanisms of EEHV pathogenesis.

Dr. Kiso adds liquid nitrogen to a tank of frozen cells.

Barack is an Asian elephant born from artificial insemination.

Dr. Kiso looks at cells under a microscope.

PHOTOS BY FELD ENTERTAINMENT, INC.

RINGLING BROS. AND BARNUM & BAILEY

ENSURING THE FUTURE OF ELEPHANTS

"The North American elephant population is not self-sustaining," says Dr. Wendy Kiso, Research and Conservation Scientist at the *Ringling Bros. and Barnum & Bailey Center for Elephant Conversation*. "Reproduction must increase several-fold to ensure the endangered Asian elephant does not become geographically extinct in North America." For these reasons, much of Dr. Kiso's work in elephant conservation has focused on cryopreservation and artificial insemination (AI). "Although natural breeding is ideal, artificial insemination has become a valuable reproductive tool for it allows females to still take part in breeding when they may not have access to a breeding (or unrelated) male. More importantly, AI allows us to genetically manage breeding opportunities so that we can ensure the elephant population remains genetically diverse and robust."

Dr. Kiso has a considerable background in environmental, zoological, and conservation sciences. Upon completion of her Bachelor of Sciences at the University of California, Irvine, she attended the Exotic Animal Training and Management (EATM) at Moorpark College in California. That study was "to get hands-on experience and learn basic husbandry and training of exotic animals," says Dr. Kiso. Following training, she obtained her Masters degree from Missouri State University in Natural and Applied Science (MNAS) with an emphasis in elephant reproduction. Dr. Kiso then completed a Doctorate in Environmental Science and Public Policy at George Mason University, in affiliation with the Smithsonian Conservation Biology Institute.

Through her work with *Ringling Bros.*, Dr. Kiso has aimed research towards the conservation and preservation of the endangered Asian elephant. *Ringling Bros.* is dedicated to this work, she says, because "the ability to cryopreserve Asian elephant sperm, and to ultimately preserve their genetics for thousands of years is invaluable, especially since the Asian elephant is threatened with extinction." *Ringling Bros.* has established the first genetic resource bank solely dedicated for Asian elephants. The Genome Resource Bank (GRB) is housed at the *Ringling Bros. Center for Elephant Conservation*, as well as at Missouri State University, storing frozen sperm (> 2000ml) from multiple male Asian elephants from *Ringling Bros.*, as well as from several other elephant bulls from across the US. The GRB, including all supplies, materials and staff, is funded entirely by Feld Entertainment, Inc., the parent company of *Ringling Bros. and Barnum & Bailey Circus*.

The goal of the Bank is to include genetic material from Asian elephant males from around the world. The biggest challenge for the GRB has been to consistently obtain high quality sperm: semen from both Asian and African elephants can be highly variable, and many ejaculates exhibit low sperm motility. This variability, including varying characteristics such as motility, concentration, and seminal plasma constituents, could be due to collection methods, which typically utilize rectal massage. Alternate methods of semen collection are being investigated by *Ringling Bros.*, says Dr. Kiso, "in hopes to enhance ejaculate quality and consistency." Another variable that may affect these characteristics is the high testosterone "rut-like" period called *musth*, which occurs without season or synchrony. Little is understood about this physiological state, and how it may influence the quality and traits of semen production. The collection of consistently high-quality semen is a vital component of Dr. Kiso's work, and research has focused on what characteristics result in better outcomes. "In my doctorate research, we found that better quality sperm were found in ejaculates in low sperm concentration with high volume," she says. "In other words, the more seminal plasma (i.e. higher volume) in the ejaculate, the better the sperm quality. We believe that seminal plasma plays a huge role in enhancing sperm motility and viability. More significantly, after extensive seminal plasma protein research, we found the presence of a protein called lactotransferrin in 85% of ejaculates exhibiting good motility."

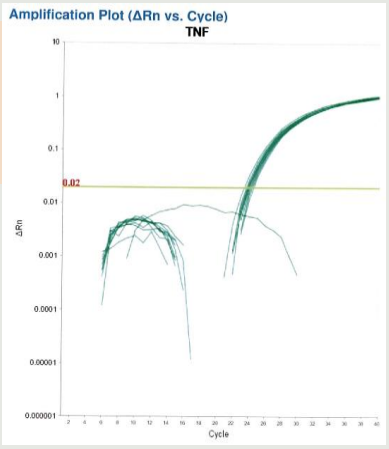
Ultimately, cryopreservation and artificial insemination have yielded promising results. A reliable method to cryopreserve Asian elephant semen has resulted in up to a 90% post-thaw survival rate. Over 40 elephant calves have been born globally from artificial insemination, through the use of chilled fresh semen in both Asian and African elephants. However, challenges still remain. While live African elephant calves have been born in Europe through the use of artificial insemination using cryopreserved semen, "we are still anxiously waiting for the first Asian elephant birth using frozen semen," says Dr. Kiso. Furthermore, much remains unknown about the elephant oocyte, and how this germ cell differs between Asian and African elephants. Nevertheless, *Ringling Bros.* has had wild success with the GRB, and "…the ability to successfully freeze Asian elephant sperm is a great success," says Dr. Kiso, "and *Ringling Bros.* continues to be a leader in this research by optimizing cryopreservation techniques."

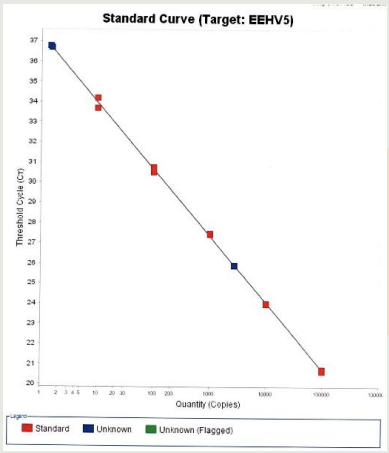
Real-Time qPCR Testing in the EEHV Laboratory at the National Zoo

Thanks to a generous donation from the International Elephant Foundation of an Applied Biosystems StepOnePlus real-time Polymerase Chain Reaction (qPCR) machine, the National Elephant Herpes Laboratory (NEHL) has been able to offer EEHV qPCR testing for diagnostics (using blood from acute cases and tissues from necropsies) and screening (testing weekly blood samples and trunk washes). The StepOnePlus allows us to quantitate the viral DNA present in blood samples, which gives valuable information to the clinician and can inform treatment decisions. Levels of EEHV in the trunk washes can also be determined, although even high levels of EEHV in trunk secretions do not necessitate treatment.

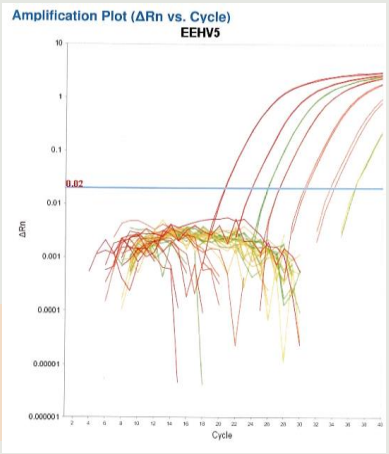
Following are some of the graphs produced by the machine and what we do with them:

For quality control, we test every sample with primers/probe for Tumor Necrosis Factor (TNF). TNF is an elephant gene that should be present if the DNA preparation went according to plan and there are no PCR inhibitors present. Any elephant DNA sample should have a detectable TNF signal (Ct of 22-25 for blood, 30s for trunk washes).





Known amounts of synthetic pieces of EEHV DNA are diluted and assayed with the EEHV primer/probes. A standard curve is generated and is used to calculate the viral genome equivalents (VGE) per reaction and per ml blood. There are several quality parameters that the standard curve has to meet in order to be used for quantitation.



The cut-off threshold for a negative sample is 0.02. When a sample is positive, there will be a logarithmic curve that goes above the threshold, when the ΔRn is plotted vs the cycle number. This plot shows the curves for five points on the standard curve (maroon curves) and two samples from the blood of an elephant calf with EEHV5 (green curves).

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- Point Defiance Zoo & Aquarium
- Ringling Bros. and Barnum & Bailey
- The Rosamond Gifford Zoo at Burnet Park
- The Saint Louis Zoological Park
- Smithsonian's National Zoo and Conservation Biology Institute
- The Tulsa Zoo
- Utah's Hogle Zoo
- Woodland Park Zoo