**1. International Elephant Foundation Project Report Cover Sheet**

**Project Title:** Detection of elephant endotheliotropic herpesvirus (EEHV)1A DNA using in situ hybridization

**Report Type:** Final

**Principle Investigator:**
Jennifer (Jaime) A. Landolfi, DVM, PhD, Dipl ACVP
Clinical Assistant Professor
University of Illinois Zoological Pathology Program
c/o Chicago Zoological Society
3300 Golf Road
Brookfield, IL 60513
(312) 585-9050
landolfi@illinois.edu

**Co-investigators:**
Paul D. Ling, PhD
Associate Professor (Tenured)
Department of Molecular Virology and Microbiology
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
pling@bcm.edu

Lauren L. Howard, DVM, Dipl. ACZM
Associate Director, Veterinary Services
San Diego Zoo Safari Park
15500 San Pasqual Valley Road
Escondido, CA 92027-7017
lhoward@sandiegozoo.org

**Project start date:** 03/2020

**Anticipated completion date:** 01/2021

*Work on the project began in 03/2020, and a 6-month time was anticipated for completion. Constraints incurred due to the SARS CoV-2 pandemic prohibited normal progress given restrictions on travel limiting access to the laboratory and interaction between investigators.*

**2. Conservation needs and summary of project progress and results:**

Elephant endotheliotropic herpesvirus (EEHV) causes EEHV hemorrhagic disease (EEHV-HD), a condition responsible for significant mortality in young Asian and African elephants. Disease is reported in both wild and zoo animals, and in susceptible individuals, infection manifests as an acute, multisystemic, often fatal hemorrhagic syndrome. To date, more than 50 confirmed fatal cases of EEHV-HD have occurred in North American and European zoos. Importantly, the disease is not limited to elephants in managed care. Several cases have been documented in free-ranging, managed, and working elephant calves in range countries including India, Thailand, Laos, Sumatra, and Nepal. Infectious disease, in general, can be a significant threat to both *ex* and *in situ* conservation efforts and species survival. Considering the majority of EEHV-HD cases have occurred in calves, infection and disease have profound implications for elephant population growth and sustainably. North America currently has 22 Asian and 52 African elephants within the age range considered at-risk for death or illness due to EEHV. An improved understanding of pathogenesis has the potential to inform management and lead to novel prevention and treatment approaches to ensure these animals survive to adulthood.

In adult elephants, EEHVs are nearly ubiquitous and behave as endogenous, host-adapted herpesviruses that do not cause clinical disease. What remains to be determined is why, in some calves, the immune system fails to control infection of the endogenous viruses resulting in hemorrhagic disease. To further understanding of EEHV-HD pathogenesis, it is necessary to visualize virus in lesional tissue to determine viral tissue and cellular tropism. Tropism is a feature of pathogenesis that impacts mechanisms of infection, dissemination, latency and shedding. Visualization of virus within lesional tissue can also provide a means of definitive diagnosis in EEHV-HD cases. The current study utilized a novel *in situ* hybridization (ISH) technique (RNAScope®) to detect and localize EEHV1A in Asian elephant tissues with EEHV-HD lesions. RNAScope® offers enhanced specificity and sensitivity as compared with conventional protocols especially in archival samples.

All work has been completed:

- All samples (archival formalin-fixed, paraffin-embedded samples of tongue and heart from EEHV1A positive cases [N=4] and negative cases [N=2]) were obtained and necessary recuts prepared.
- All ISH probes (Asian elephant beta actin [positive control], EEHV1A DNA polymerase and terminase) were designed and synthesized.
- The RNAScope® ISH protocol was validated for use with the archival elephant tissue using the positive control beta actin probe in all samples. Successful hybridization was evidenced by discrete red nuclear hybridization signal in all samples (Figs. 1 and 2). These results confirmed the archival study materials were adequate for assay.
- All samples were hybridized with the EEHV1A DNA polymerase and terminase probes. In the positive cases, red hybridization signal was readily evident in endothelial cell nuclei in both the heart and tongue for both probes. Generally, a higher number of heart endothelial cell nuclei had hybridization signal as compared with tongue. No hybridization signal was present in heart or tongue sections from the negative control animals (Figs. 3 and 4).
- In positive cases, hybridization signal was compared between the DNA polymerase and terminase probes. Analyses were attempted using image analysis software (ImageJ; imagej.nih.gov), however, interstitial hemorrhage within sections due to EEHV-HD interfered with the software's ability to accurately distinguish the red hybridization signal. To ensure accuracy, analyses were conducted manually. For each case and each tissue (heart and tongue), forty, 400x magnification images were captured, and the absolute number of endothelial cell nuclei with positive red hybridization signal per image was counted. Counts were then averaged to provide the mean number of positive endothelial cell nuclei for each animal and tissue. Counts were also normalized by calculating the proportion of total nuclei (endothelial and cardiomyocyte) per image with positive hybridization signal. In all cases, a higher number of endothelial cell nuclei in heart had signal than in tongue for both probes (Figs 5

and 6). When comparing the two probes, the same trend was evident in both heart and tongue. Hybridization with the terminase probe resulted in detection of higher absolute and normalized number of endothelial cell nuclei as compared with the DNA polymerase probe (Figs 7-10). Results were evaluated using a Student's t test. For the tongue, the difference in values for the two probes was statistically significant (absolute values, $p = 0.045$; normalized values, $p = 0.044$). Statistical significance could not be demonstrated for the heart, however, this may have been a consequence of overall low sample size of the study. Altogether, findings indicated that the terminase probe had greater sensitivity, and thus would be sufficient alone and superior to the DNA polymerase probe for detecting EEHV1A DNA in archival tissues from cases of EEHV-HD.

**3. Project goals and objectives and associated actions:**

Project goals/objectives were to:

1. Identify viral nucleic acid in lesional tissues from archival EEHV-HD cases due to EEHV1A in Asian elephants using RNAScope® in situ hybridization

Towards this objective, the RNAScope® ISH protocol was validated for use in archival formalin-fixed, paraffin-embedded elephant tissue. All study samples were hybridized with the beta actin positive control probe, and positive nuclear signal was present in all tissues from both EEHV1A positive and negative cases (Figs. 1 and 2). These results indicated samples were adequate for assay with the EEHV1A DNA polymerase and terminase probes. The EEHV1A DNA polymerase and terminase probes were then utilized to identify viral nucleic acid in lesional tissues (heart and tongue) of the EEHV1A positive cases as well as to screen the negative cases for probe specificity. Lack of signal in tissues from the negative control cases provided evidence of specific hybridization (Figs. 3 and 4). In the positive cases, successful hybridization was evidenced by red signal in the endothelial cell nuclei of both heart and tongue. Importantly, nonspecific background signal was negligible in all cases.

2. Determine if targeting the viral DNA polymerase or terminase gene alone is sufficient to detect EEHV1A and demonstrate which gene represents the optimal target for EEHV1A detection in archival cases of EEHV-HD due to EEHV1A in Asian elephants using RNAScope® in situ hybridization

To address this objective, hybridization signal in the positive cases was compared between the DNA polymerase and terminase probes. To ensure accuracy, analyses were conducted manually. For each case and each tissue (heart and tongue), forty, 400x magnification images were captured, and the absolute number of endothelial cell nuclei with positive red hybridization signal per image was counted. Counts were then averaged to provide the mean number of positive endothelial cell nuclei for each animal and tissue. Counts were also normalized by calculating the proportion of total endothelial cell nuclei as a function of total nuclei (endothelial and cardiomyocyte) per image with positive hybridization signal. When comparing the two probes, the same trend was evident in both heart and tongue. Hybridization with the terminase probe resulted in detection of higher absolute number and proportion of total nuclei as compared with the DNA polymerase probe (Figs 7-10). Differences in values between terminase and polymerase probes were statistically significant for the tongue. Results indicated that the terminase gene is the optimal target for EEHV1A detection in archival cases of EEHV-HD using RNAScope® ISH.

**4. Differences from original proposal:**

The timeline for the project was impacted by laboratory restrictions related to the SARS CoV-2 pandemic.

Samples from additional EEHV1A positive cases (N=2) became available and were added to project scope in order to improve power. Conservative use of RNAScope® ISH reagents allowed for assay of these additional samples with the available stock, thus no

additional funding was required to support this modification of the project. The total cases included in the study now was four EEHV1A positive cases and two negative cases.

For completion of Objective 2, assessment was attempted using image analysis software (ImageJ; imagej.nih.gov), however, interstitial hemorrhage within sections due to EEHV-HD interfered with the software's ability to accurately distinguish the red hybridization signal. To ensure accuracy, analyses were conducted manually as described above.

**5. Conservation outcomes, findings and accomplishments to date:**

Results have provided validation of a new ISH technique (RNAScope®) for use in archival formalin-fixed paraffin embedded elephant tissues. The application of RNAScope® in elephants is not limited to the diagnosis and study of EEHV. Future work using novel elephant probes for immune markers could provide insights into the host response in a variety of diseases and conditions. Using other elephant pathogen-specific probes, the technique could also be used for confirmatory diagnosis of important elephant diseases (e.g. *Mycobacterium tuberculosis*) that impact the welfare and conservation of these endangered species.

Results also illustrate the usefulness of this technique for the investigation of viral tissue and cellular tropism in EEHV-HD. Future, related work is planned to examine a wide variety of archival tissues from additional EEHV-HD cases with RNAScope® ISH to define EEHV1AI tissue and cellular tropism. This will be a key step towards understanding disease pathogenesis. Importantly, this study also provided validation of a new technique to facilitate definitive diagnosis of EEHV1A infection in cases of EEHV-HD.

**6. Human and/or elephant impact:**

North America currently has 22 Asian and 52 African elephants within the age range considered at-risk for death or illness due to EEHV. An improved understanding of pathogenesis has the potential to inform management and lead to novel prevention and treatment approaches to ensure these animals survive to adulthood. Advancements made towards the improved management of this devasting disease in captive populations will be directly applicable to designing intervention strategies to curb the impact of the disease in range countries.

**7. Problems with the project:**

The only significant obstacle encountered was delay in work related to restrictions imposed by the SARS CoV-2 pandemic. No impediments to project vision or methodology occurred.

**8. Project success and short- and long-term goals:**

All planned work was completed with successful outcomes. Based upon the encouraging results of this study, short-term future goals include a complete tropism study that will use RNAScope® to evaluate a wide variety of tissues from all available archival EEHV1A cases in order to determine tissue and cellular tropism. Proposals are currently pending to provide financial support for this work. Another short-term goal will be to make an RNAScope® assay for the definitive diagnosis of EEHV1A in archival samples commercially available to the international elephant community.

Long-term goals will relate to application of knowledge gained regarding pathogenesis of EEHV to impact calf survival. Currently, susceptibility to EEHV-HD in elephant calves is poorly understood. Identifying factors that influence susceptibility and devising methods to combat them is one route towards ensuring greater calf survival. Defining the viral tissue and cellular tropism is the first step in understanding pathogenesis of disease. Tropism provides the basis for pathogenic mechanisms such as infectivity, dissemination, shedding and latency. These disease dynamics have the potential to directly impact susceptibility. Another route towards improved calf survival is efficacious treatment in the face of disease. Current treatment regimens are largely supportive, empirical and/or anecdotal. Importantly, similar regimens have produced variable results in clinical cases. Understanding how a pathogen causes damage can facilitate development of methods to prevent or counteract that damage. Investigating the tissue and cell types that are infected in cases of active EEHV-HD indicates sites of potential insult. Such

knowledge can be utilized to direct development and application of both targeted and supportive therapeutic interventions that may enhance survival in affected calves.

**9. Project "next steps" and implication for future conservation goals:**

See short- and long-term goals above.

**10. Human interest story:**

In addition to vital disease research relevant to humane management and conservation of elephants, this project provided the opportunity for graduate student (Kirstin Cook, University of Illinois Department of Veterinary Clinical Medicine) educational advancement. This study was the focus of Dr. Cook's research in pursuit of a Master of Science degree. Involvement with the project provided Dr. Cook with practical experience and nurtured development of essential skills necessary for future directed investigations into animal disease.

**11. Associated organizations and their roles:**

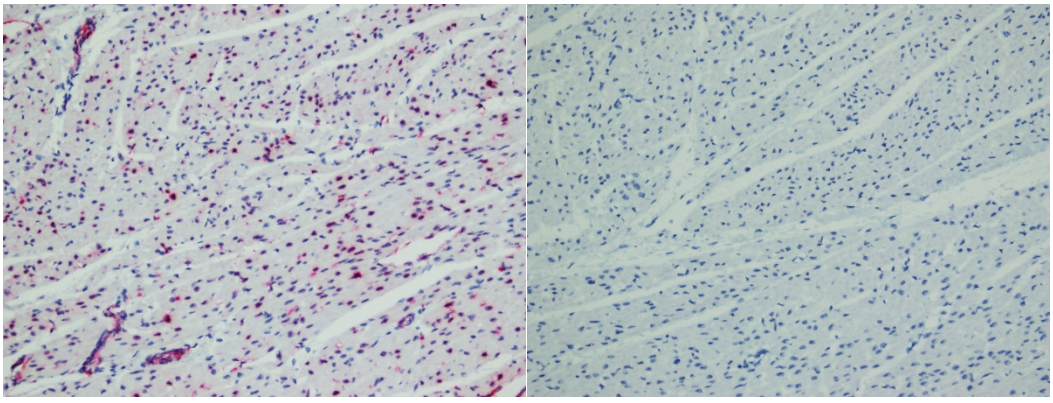
The University of Illinois Zoological Pathology Program (ZPP) is the PI's (Landolfi) and graduate student's (Cook) home department. ZPP has provided the laboratory space and necessary equipment for the project. ZPP's support has also facilitated both Dr. Landolfi's and Cook's involvement in the project.

Both San Diego Zoo Global and Baylor College of Medicine supported participation of Drs. Howard and Ling, respectively, with the project.

**12. Itemized financial report: See separate page**

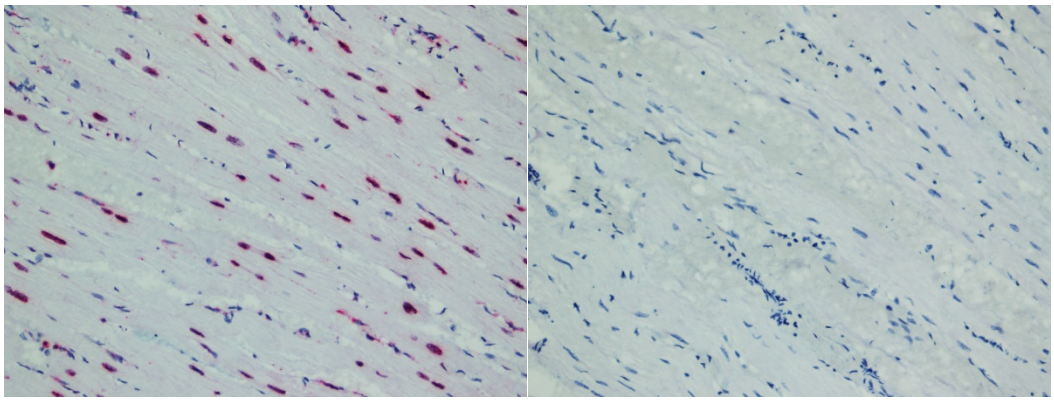
**13. Images:**

**Figure 1**: RNAScope® in situ hybridization (ISH) of Asian elephant beta actin in EEHV1A negative case



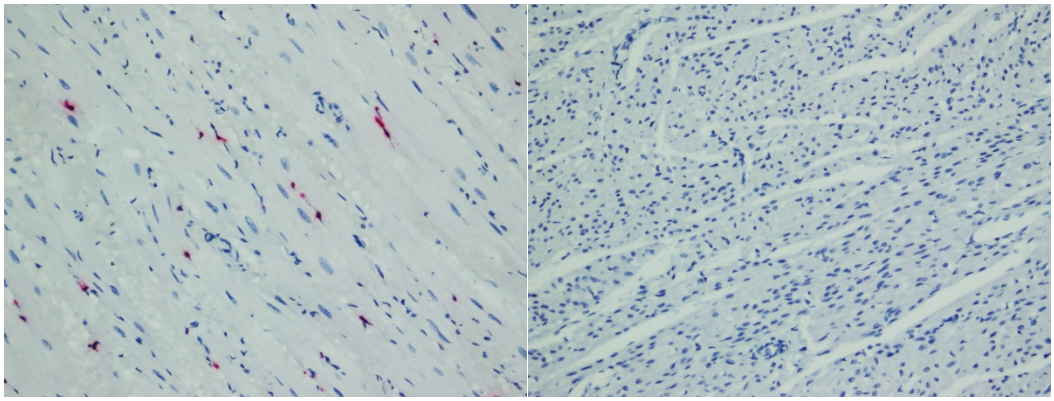
**Legend:** The image on the left depicts a section of heart from an EEHV1A negative elephant with abundant positive (red) nuclear hybridization signal for beta actin. The image on the right is the same section hybridized with the negative control probe; note lack of red hybridization signal.

**Figure 2**: RNAScope® in situ hybridization (ISH) of Asian elephant beta actin in EEHV1A positive case



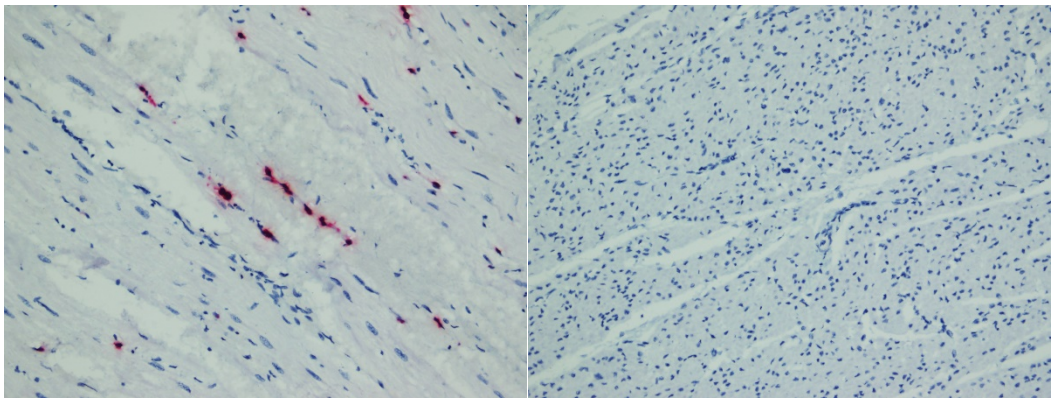
**Legend:** The image on the left depicts a section of heart from an EEHV1A positive elephant with abundant positive (red) nuclear hybridization signal for beta actin. The image on the right is the same section hybridized with the negative control probe; not lack of red hybridization signal.

**Figure 3:** RNAScope® ISH of EEHV1A DNA polymerase



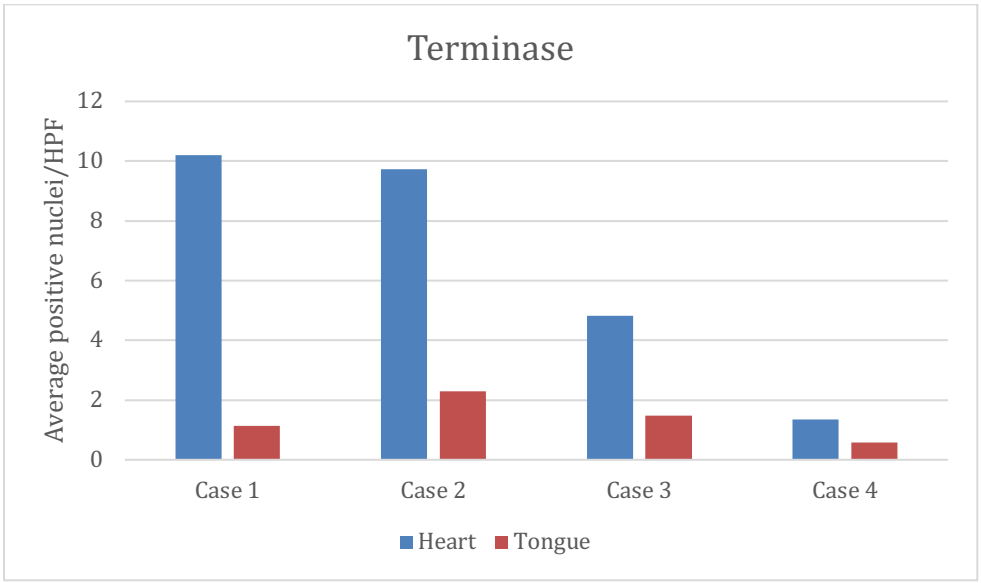
**Legend:** The image on the left is a section of heart from an EEHV1A positive case; endothelial cells have positive (red) signal. The heart from an EEHV negative case depicted in the image on the right and lacks positive (red) signal.

**Figure 4**: RNAScope® ISH of EEHV1A terminase



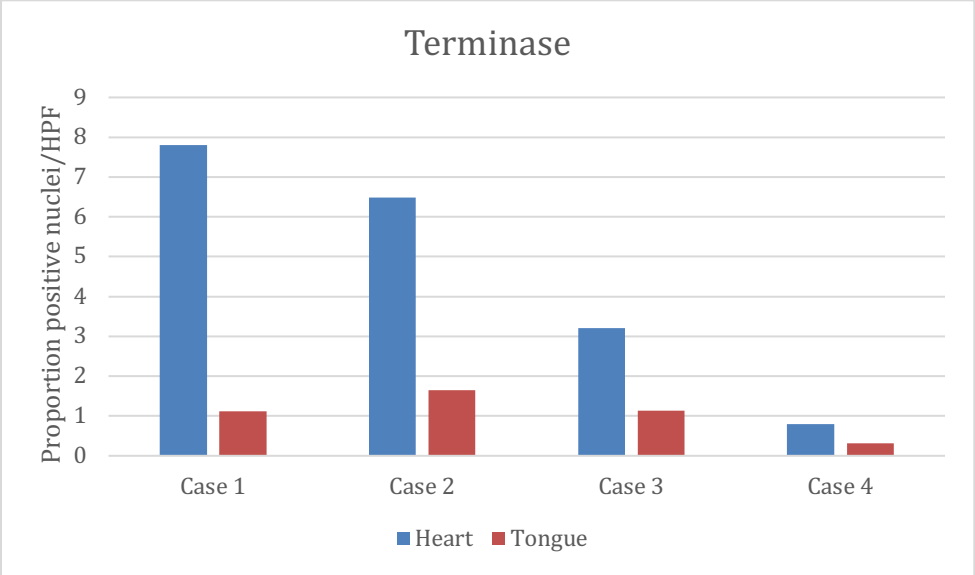
**Legend:** The image on the left is a section of heart from an EEHV1A positive case; endothelial cells have positive (red) signal. The heart from an EEHV negative case depicted in the image on the right and lacks positive (red) signal.

**Figure 5**: RNAScope® ISH of EEHV1A terminase in heart vs. tongue



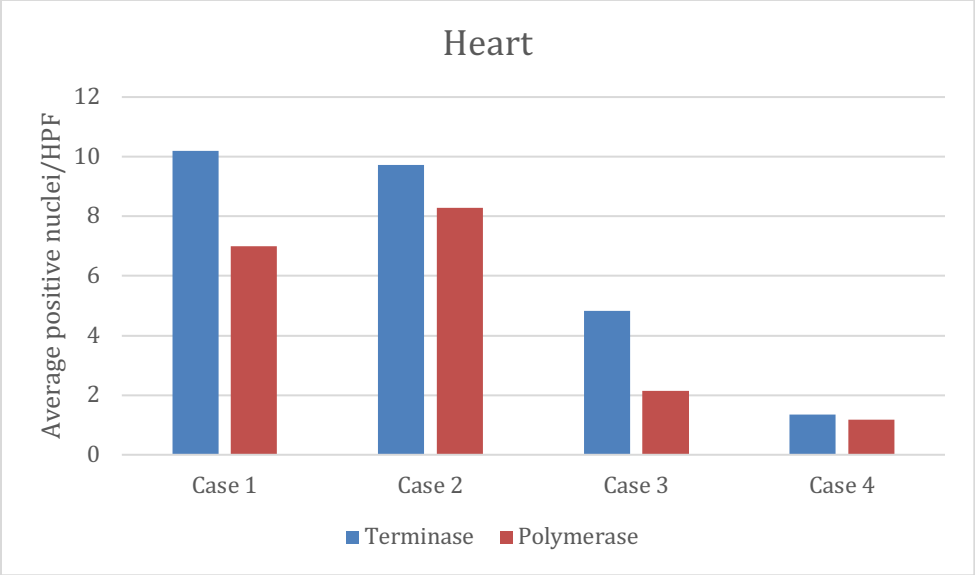

**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase probe as determined by manual counting of the absolute number of positive endothelial cell nuclei in forty 400x fields.

**Figure 6**: RNAScope® ISH of EEHV1A terminase in heart vs. tongue



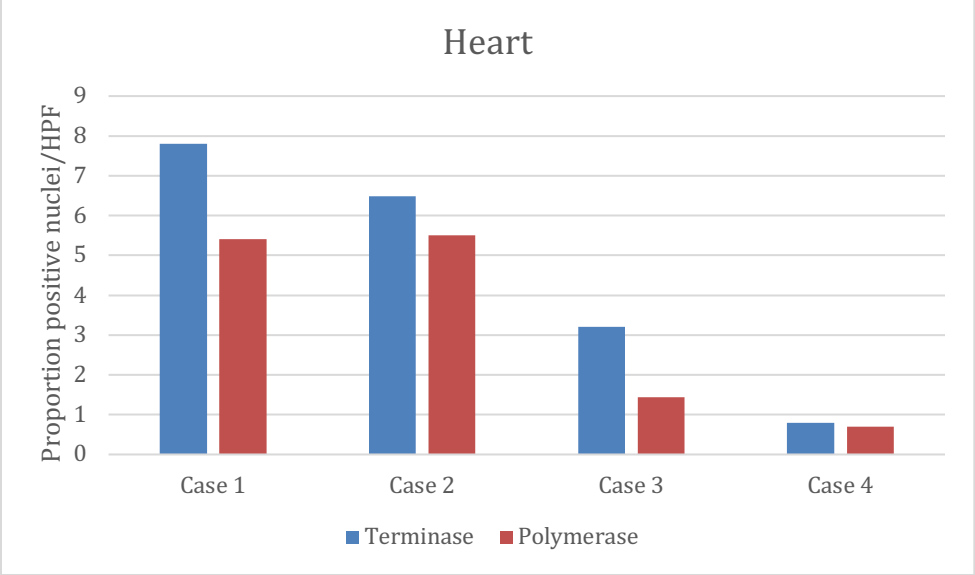

**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase probe as determined by manual counting of the number of positive endothelial cell nuclei normalized to the total number of nuclei (endothelial and cardiomyocyte) in forty 400x fields.

**Figure 7**: RNAScope® ISH of EEHV1A terminase and DNA polymerase in heart



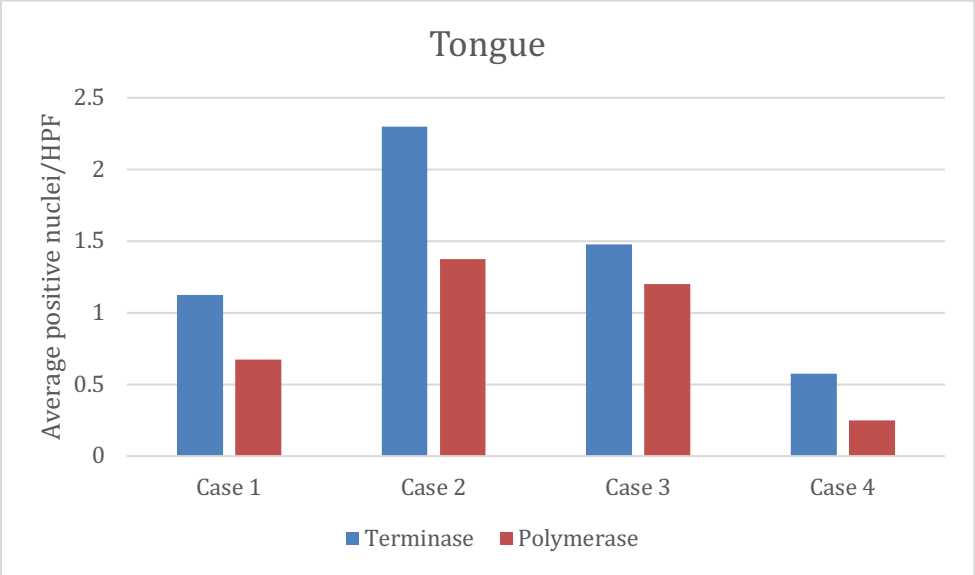

**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase (blue) and polymerase (red) probes in sections of heart as determined by manual counting of the absolute number of positive endothelial cell nuclei in forty 400x fields.

**Figure 8**: RNAScope® ISH of EEHV1A terminase and DNA polymerase in heart



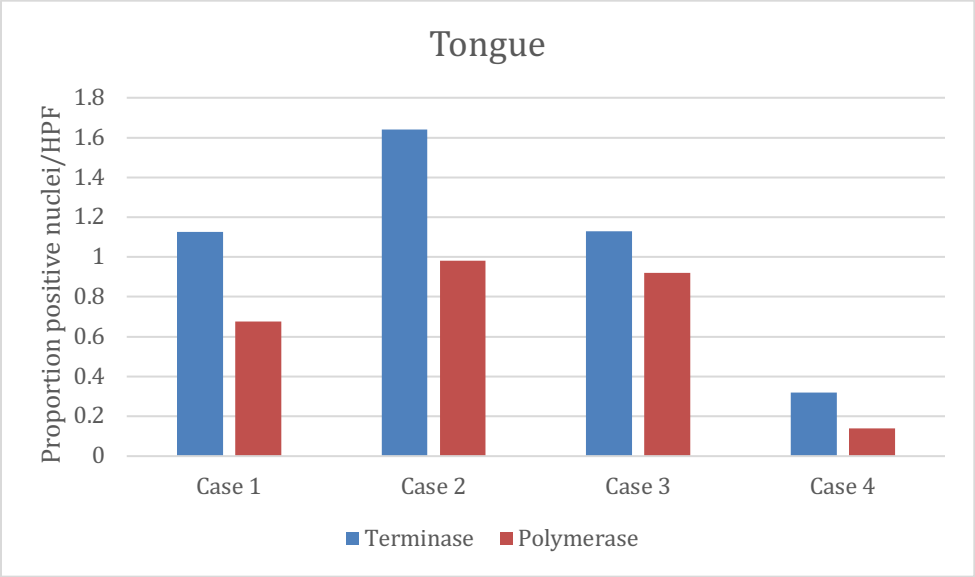

**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase (blue) and polymerase (red) probes in sections of heart as determined by manual counting of the number of positive endothelial cell nuclei normalized to the total number of nuclei (endothelial and cardiomyocyte) in forty 400x fields.

**Figure 9**: RNAScope® ISH of EEHV1A terminase and DNA polymerase in tongue




**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase (blue) and polymerase (red) probes in sections of tongue as determined by manual counting of the absolute number of positive endothelial cell nuclei in forty 400x fields.

**Figure 10**: RNAScope® ISH of EEHV1A terminase and DNA polymerase in tongue




**Legend**: The chart depicts the average number of endothelial cell nuclei with positive hybridization signal for the terminase (blue) and polymerase (red) probes in sections of tongue as determined by manual counting of the number of positive endothelial cell nuclei normalized to the total number of nuclei (endothelial and cardiomyocyte) in forty 400x fields.

**14. Video clip: see attached**

**15. Plans for publication and/or presentation:**

Results are being collated and prepared for publication in a peer-reviewed journal. Dr. Cook is heading these efforts as part of her scholarly pursuits. It is also possible that findings may be shared at future regional or national scientific conferences/meetings/workshops, though no specific plans exist to date.

**16. Media coverage:**

NA

**17. Associated websites, blogs, social media, etc:**

NA

**Itemized financial report**

| BUDGET ITEM | RECEIVED FROM IEF | SPENT | BALANCE |
|---|---|---|---|
| Project personnel | $0 | $0 | $0 |
| Travel expenses | NA | NA | NA |
| Lodging and meals | NA | NA | NA |
| Activity | | | |
| Equipment | $0 | $0 | $0 |
| Supplies:<br>• RNAscope® Custom probe design service (ACD Bio; x 3)<br>• RNAscope® Custom Asian elephant beta actin probe<br>• RNAscope® Custom EEHV1A DNA polymerase probe<br>• RNAscope® Custom EEHV1A terminase probe<br>• RNAscope® negative control probe<br>• RNAscope® ISH reagent kit (x 7)<br>• Laboratory consumables (gloves, pipette tips, molecular grade water, etc.) | • $1500<br>• $1000<br>• $1000<br>• $1000<br>• $150<br>• $5600<br>• $300 | • $1500<br>• $1000<br>• $1000<br>• $1000<br>• $150<br>• $5600<br>• $300 | • $0<br>• $0<br>• $0<br>• $0<br>• $0<br>• $0<br>• $0 |
| Services:<br>Preparation of unstained slide recuts for ISH (4 cases x 4 tissues/case = 16 samples; 16 samples x 10 recuts/sample = 160 recuts; 160 recuts x $10/recut = $1600) | $1600 | $1540.50 | $59.50 |
| Misc. expenses | $0 | $0 | $0 |
| Foregone indirects @ 24% | $0 | $0 | $0 |
| **TOTAL** | $12150 | $12,090.50 | $59.50 |