# Final Report
# Research Work Order #194
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**Project Title: Further Strategies for Evaluating the Etiological Role of a Tumor-associated Herpesvirus in Marine Turtle Fibropapillomatosis**

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## INTRODUCTION

In 1992, an interdisciplinary research team headquartered at the University of Florida began studies in key targeted areas of fibropapillomatosis (FP) etiology and pathogenesis. At that time, little was known about FP outside of field studies documenting its prevalence in different areas of the world and studies of tumor histopathology. Our primary objective was to develop a broad-based scientific understanding of FP by applying principles of tumor biology, immunology, pathology, virology, molecular biology, and epidemiology to FP in the green turtle, *Chelonia mydas*. Long-term goals included the development of assays for FP and study of any role of environmental co-factors in the disease. This report is a continuation of that effort and the results reported here bring us closer to understanding the role of a tumor-associated herpesvirus in marine turtle fibropapillomatosis.

## OVERALL SUMMARY

Sea turtle conservation efforts must include the means to monitor the health status of sea turtle populations for exposure to disease-associated microorganisms. Critically needed are improvements in diagnostics, including the development of defined pathogen-specific antigens for use in immunoassays that measure disease exposure. Herpesviruses are associated with several diseases of marine turtles including lung-eye-trachea disease (LETD) and fibropapillomatosis. While the LETD-associated herpesvirus (LETV) can be cultivated in the laboratory, efforts to cultivate the FP-associated herpesvirus (FPHV) have been unsuccessful, limiting diagnostic assay development and epidemiological studies. In this study, we have extensively studied LETV in order to gain critical information about both LETV and FPHV. In doing this we have developed a better understanding of FP and the potential role of FPHV in its etiology. This work moves us closer to the development of an assay to detect exposure of wild sea turtles to FPHV.

This research has demonstrated that marine turtle herpesviruses can persist for extended periods of time as infectious agents in the marine environment and that wild green turtles in Florida are exposed to the LETD-associated herpesvirus. This is the first description of LETV infection in free-ranging marine turtles. In addition, data is presented that supports the hypothesis that LETV and FPHV infections are independent. These data reveal new levels of complexity that must be addressed before reliable serodiagnostic assays for herpesvirus infections of chelonians can be developed for widespread application. The results reported here also raise new concerns about the potential impact of infections by new herpesviruses on populations of wild marine turtles, an area which has previously been unexplored by turtle biologists.

## RESULTS SUMMARIES

***Note**: Copies of 3 papers published or in press are attached to this report. These contain the full details of results, materials, and methods*

### 1. Persistent Infectivity of a Green Turtle Disease-Associated Herpesvirus after Exposure to Seawater

***Note: Certain aspects of this research were funded by RWO 180 to P.A. Klein as well as this RWO.***

Herpesviruses are associated with several diseases of marine turtles including Lung-Eye-Trachea disease and Gray Patch Disease (GPD) of green turtles (Chelonia mydas) and fibropapillomatosis of green, loggerhead (Caretta caretta), and olive ridley turtles (Lepidochelys olivacea). Stability of chelonian herpesviruses in the marine environment has not been previously studied. In these experiments, LETD-associated herpesvirus (LETV) was used as a model chelonian herpesvirus to test viral infectivity after exposure to seawater. The LETV was grown in terrapene heart (TH-1) cells and then virus preparations were dialyzed for 24 to 120 hr against aerated artificial or natural seawater or Hank's balanced salt solution (HBBS). TH-1 cell cultures were inoculated with seawater-exposed LETV, and on day 10 post-infection cells were scored for cytopathic effect (CPE). Virus samples tested up to 120 hr after seawater exposure were positive for the herpesvirus DNA polymerase gene by polymerase chain reaction. Electron microscopy revealed intact LETV nucleocapsids after exposure of LETV to artificial seawater or HBSS for 24 hr at 23 C. LETV preparations were then titered for infectivity and were found to remain infectious after 120 hr of exposure to natural and artificial seawater at 23 C. Similar results were obtained with a second cultivable chelonian herpesvirus, HV2245. LETV infectivity could not be detected after 48 hr exposure to artificial seawater at 30 C. Since LETV remains infectious for extended periods of time in the marine environment, it is possible that FP-associated and GPD-associated herpesviruses also may be stable. These findings are significant both for researchers studying the epidemiological association of herpesviruses with diseases of marine turtles and for individuals who handle turtles in marine turtle conservation efforts.

### 2. Detection of Antibodies to a Disease-associated Herpesvirus of the Green Turtle, *Chelonia mydas*

Lung-eye-trachea disease-associated herpesvirus—is linked with morbidity and mortality in mariculture-reared green turtles, but its prevalence among and impact on wild marine turtle populations is unknown. An enzyme-linked immunosorbent assay (ELISA) was developed for detection of anti-LETV antibodies and could distinguish LETV exposed green turtles from those with antibodies to fibropapillomatosis-associated herpesvirus. Plasma from two captive-reared green turtles immunized with inactivated LETV served as positive controls. Plasma from 42 healthy captive-reared green turtles, and plasma from 30 captive-reared green turtles with experimentally induced fibropapillomatosis (FP) and anti-FPHV antibodies had low ELISA values on

LETV antigen. A survey of wild green turtles with (n=19) and without FP (n=27) (with and without anti-FPHV antibodies, respectively) identified individuals with antibodies to LETV regardless of their FP status. The seroprevalence of LETV infection was 13%. The presence of antibodies to LETV in plasma samples was confirmed by Western blot and immunohistochemical analyses. These results are the first to suggest that wild Florida green turtles are exposed to LETV or to antigenically closely related herpesvirus(es) other than FPHV and that infection with FPHV and LETV are most likely independent events. This is the first ELISA developed to detect antibodies for a specific herpesvirus infection of marine turtles. The specificity of this ELISA for LETV (ability to distinguish LETV from FPHV) makes it valuable for detecting exposure to this specific herpesvirus and enhances our ability to conduct seroepidemiological studies of these disease-associated agents in marine turtles.

### 3. Survey of Florida Green Turtles for Exposure to a Disease-associated Herpesvirus

A recently developed enzyme-linked immunosorbent assay (ELISA) was used to assess exposure of Florida wild green turtles, *Chelonia mydas*, to LETV, the herpesvirus associated with lung-eye-trachea disease. Plasma samples from 329 wild juvenile green turtles netted in the Indian River lagoon, along the Sebastian reef, or in the Trident basin (Indian River and Brevard Counties, Florida, USA) were tested by ELISA for the presence of antibodies to LETV. Plasma samples from 180 wild juvenile green turtles were tested from these study sites to compare the prevalence of anti-LETV antibodies. While some plasma samples from each site contained anti-LETV antibodies (confirmed by Western blot analysis), plasma samples collected from the Indian River lagoon had statistically higher optical density values measured in the ELISA. No statistical differences were observed when these same plasma samples were analyzed for changes in the level of anti-LETV antibodies over three years (1997, 1998, and 1999). To explore the relationship between anti-LETV antibodies and fibropapillomatosis (FP), plasma from 133 green turtles scored for fibropapilloma tumor severity were tested by ELISA. There was no correlation between tumor severity and the presence of antibodies against LETV. Additional plasma samples collected from 16 tagged green turtles captured and sampled more than once (recaptures) were also tested to monitor antibody levels to LETV relative to the FP status of individual turtles over time. Again there was no clear relationship between FP tumor status and the presence of antibodies to LETV. Finally, ELISA tests on plasma from 13 nesting female turtles (9 green and 4 loggerhead) revealed high levels of anti-LETV antibodies in 11 individuals, including 2 loggerhead turtles. These results provide strong evidence that wild Florida green turtle populations at these three study sites are exposed to LETV or a closely related virus and that loggerhead turtles may be exposed as well. Based on a cutoff optical density value of 0.310, 71 out of the 329 wild Florida green turtles tested were seropositive for LETV antibodies (seroprevalence = 21.6%). In addition, no relationship between FP tumor severity or status and the presence of anti-LETV antibodies was found, further supporting the

hypothesis that LETV and the FP-associated herpesvirus are separate infections of marine turtles.

## 4. Development of a LETV genetic library

Terrapene heart cells ($1.0X10^7$) were infected with LETV clone 221. After 6-7 days post infection, cells with 100% cytopathic effect were collected from the supernatant by centrifugation 2500 g 10 min at 4 °C. Infected pelleted cells were resuspended in L buffer (0.1M EDTA pH 8.0, 0.01 M Tris HCl pH 7.6, 0.02M NaCl), mixed with equal volume 1.2% low temperature agarose, and poured in plug mold apparatus. Embedded infected cells were lysed *in situ* with 2 changes of L buffer plus 1% v/v Sarkosyl plus 0.1% w/v proteinase K for 48 hrs at 50 C. After 3 washes in L buffer, plugs were kept in TE pH 8.0 at 4 °C. A 10 well 1.0% 0.5X TBE low temperature agarose gel was poured and _ of a plug was loaded into each of 8 wells. Lambda Ladder PFG marker (New England Biolabs, Beverly MA) and Low Range PFG Marker (New England Biolabs, Beverly MA) were loaded into the remaining wells. LETV 221 genomic DNA was separated by pulse-field gel electrophoresis (200V, 24 hrs, 50-90sec) at 4 C. The gel was stained in ethidium bromide bath and bands visualized on long wavelength UV box. Bands corresponding to the LETV genome (approx 120 kb) were cut out of the gel and were melted at 70 C for 20 min. Melted gel slices were digested in β agarase (5 units/ 100 mg) overnight at 45 C. The mixture was transferred to Centricon YM-100 (Millipore, Medford MA) and concentrated by centrifugation at 500 g for 20 min @ room temperature. The centricon was washed with 20% isopropanol, reconcentrated, and then washed with 2 volumes of TE pH 7.5. The centricon was inverted and the DNA sample was collected by centrifugation. 20 μg of LETV 221 DNA was sonicated to generate fragments 1-2 kb in size and was size fractionated on CHROMA SPIN-100 gel filtration columns (Clontech, Palo Alto CA) to remove DNA molecules smaller than 300 bp. The DNA was eluted, ethanol precipitated, and blunt ends were formed using T4 DNA polymerase. The DNA was ligated into SmaI linearized pUC18 cloning vector and then the ligated product was ethanol precipitated. The ligated product was electroporated into E. coli host TOP10 and clones were blue/white selected on LB plates plus X-gal and IPTG. Clones were grown in LB plus ampicillin for 20 hours in 96 well plate format with agitation. Bacterial clones were harvested and DNA extracted with Qiagen Biorobot (Qiagen Valencia CA). DNA samples were quantified, and then sequenced on MegaBACE sequencer using ET terminator mix in MJ thermocycler (Amersham Pharmacia, Biotech, Piscataway NJ). Sequences were compared to other sequences by blastx (basic local alignment search tool in protein database) searching the NCBI (National Center for Biotechnology Information) database. Clones matching herpesvirus genes were arranged according to the gene order of the model alpha-herpesvirus, human simplex virus 1.

### 5. Identification of immunodominant LETV antigens

We have initiated studies to identify the specific LETV proteins that green turtles respond to with antibody formation following infection with this herpesvirus. We used two-dimensional Western blots to study a 38 kD protein that is recognized by antibodies in the plasma of green turtles exposed to LETV. Figure 1 shows that this protein can be resolved and visualized by two-dimensional gel electrophoresis (Figure 1 A) and Western blotting (Figure 1B). This protein is currently being sequenced in the Protein Core of the University of Florida Biotechnology Program.

## DISCUSSION

Seroepidemiology, which uses a variety of laboratory-based serological assays, can determine whether an individual has been infected with the disease-associated microorganism and how many individuals in a population are infected (seroprevalence of the disease). Seroepidemiology can track the spread of an infection through a population or to new populations that have previously been disease free. Seroepidemiology can help to determine the routes of disease spread and identify mechanisms of disease transmission (vectors, high risk environmental conditions, etc.).

Seroepidemiology can also be used to provide evidence which links infection with an infectious agent and the development of the full blown clinical disease. It is especially useful in cases where the infectious agent cannot be isolated and grown in culture for use in transmission studies as is currently the case for FP. The seroepidemiological approach is the cornerstone of Hill's Criteria for Disease Causation. This seroepidemiological approach has been used successfully in human diseases in which transmission studies are not feasible, such as Kaposi's sarcoma, a cancer syndrome involving infection with a newly identified herpesvirus.

The critical factor in using seroepidemiology as a tool to monitor and study infectious disease in populations is having reliable, sensitive, and specific serological assays that can detect antibodies against the infectious agent. Such assays require two main components; antigens from the microorganism and specific secondary antibodies which can detect the primary anti-infectious agent antibodies in plasma samples from animals. Previously we have developed unique monoclonal antibody reagents against marine turtle IgY, IgM, and 5.7s immunoglobulin subclasses as secondary antibodies. These can detect turtle antibody responses to infectious agents. The research described here will lead to the development of pathogen-specific recombinant proteins and antigenic peptides (antigens) of both the LETV and FPHV for use in seroepidemiological studies of these two diseases of marine turtles.

## PRESENTATIONS AND PUBLICATIONS FROM THIS PROJECT

### Presentations.

Curry, S. S., D. R. Brown, E. R. Jacobson, and P. A. Klein. Persistent infectivity of Chelonian herpes viruses after exposure to artificial seawater. In Proceedings of the Nineteenth Annual Symposium on Sea Turtle Biology and Conservation, March 2-5, 1999, South Padre Island, Texas. U.S. Dep.Commer. NOAA Tech. Memo. NMFS-SEFSC., In Press.

Origgi, F.C., Jacobson, E., Herbst, L.H., Klein, P.A., and Curry, S.S.. Development of Serological Assays for herpesvirus Infections in Chelonians. 20th Annual Symposium on Sea Turtle Biology and Conservation, Orlando, Fl, February 29th-March 4th, 2000, In press.

Coberley, S.S., Herbst, L.H, Ehrhart, L.M., Bagley, D.A., Hirama, S., Shaf, S.A., Moretti, R.H., Jacobson, E.R., and P.A. Klein. Serological Detection of Herpesvirus Infections in Green Turtles. Abstract 267.7, The FASEB Journal, Part I, Abstracts 2.1-537.42, Experimental Biology 2001, March 31-April 4th, 2001.

Coberley, S.S., Herbst, L.H, Ehrhart, L.M., Bagley, D.A., Hirama, S., Shaf, S.A., Moretti, R.H., Jacobson, E.R., and P.A. Klein. Serological Detection of Herpesvirus Infections in Green Turtles. 21st Annual Symposium on Sea Turtle Biology and Conservation, Philadelphia, PA February 23rd-February 28th,2001, In press.

Coberley, S., Herbst, L.H., Ehrhart, L., Bagley, D., Hirama, S., Schaf, S., Moretti, R.,Jacobson, E., Condit, R. and P. Klein. Detection of Antibodies to a Disease-associated Herpesvirus of the Green Turtle, *Chelonia mydas*. American Society for Virology. Madison, Wisconsin. July 21-25th, 2001.

### Publications.

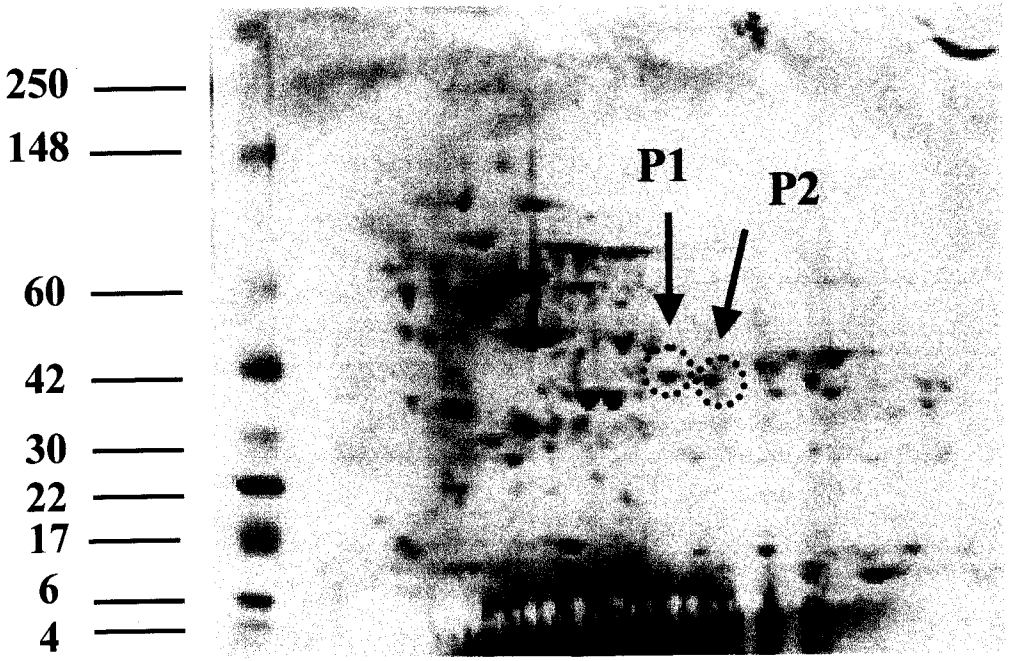
1.Curry, S. S., D. R. Brown, E. R. Jacobson, and P. A. Klein., Ehrhart, L.M., Gaskin,J.M., Blahak, S., Herbst, L.H. and P. A. Klein. , Persistent Infectivity of a Chelonian Herpesvirus after Exposure to Seawater. J. Wildlife Diseases 36, 792-797, 2000.

2. Coberley, S.S., Herbst, L.H, Brown, D.R., Ehrhart, L.M., Bagley, D.A., Schaf, S.A., Moretti, R.H., Jacobson, E.R., and P.A. Klein. Detection of Antibodies to a Disease-associated Herpesvirus of the Green Turtle, *Chelonia mydas*. J.Clinical Microbiol., 39, 3572-3577, 2001.

3. Coberley, S.S., Herbst, L.H, Brown, D.R., Ehrhart, L.M., Bagley, D.A, Hirama, S., Jacobson, E.R., and P.A. Klein. Survey of Florida Green Turtles for Exposure to a Disease-associated Herpesvirus. Diseases of Aquatic Organisms, 2001. In press.

**Figure 1. Two Dimensional Gel Electrophoresis and Western Blot of LETV Proteins**

## A. Coomassie Stained 2D Gel




## B. Western Blot