FLORIDA COOPERATIVE FISH AND WILDLIFE RESEARCH UNIT

PROJECT STATUS REPORT

TITLE: Seroepidemiological Studies of Herpesvirus-associated Diseases of Marine Turtles: Fibropapillomatosis and Lung-Eye-Trachea Disease

1.PROJECT OFFICER:

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4. RESEARCH WORK ORDER #: 213
5. FUNDING AGENCY: USFWS

6. START DATE: 07 / 01/ 2001 END DATE: 12 / 31 / 2003

7. This Reporting Period: 8-31-02 to 12-31-03 (FINAL REPORT[see full report attached))

15. REPORTING PERIOD FOR DELIVERABLES: Final report due: 12-31-03

9. ABSTRACT OF PROJECT(maximum 4000 characters):

LETV

Antigenic LETV expressed peptides will be used to develop an LETV ELISA following methods previously established for the ELISA using whole LET herpesvirus. With expressed antigen, there will be fewer limitations on the number of plasma samples that can be screened. Plasma samples previously tested by the ELISA using whole virus will be used to validate the specificity of the assay. Specifically, the plasma samples obtained from the captive-reared turtles prior to and after immunization with inactivate LETV will be used. Plasma samples from captive-reared green turtles with no known exposure to herpesviruses will serve as additional known-negative samples.

We will utilize the same plasma bank developed collaboratively with the University of Central Florida to identify marine turtles in Florida exposed to LETV. This will provide some preliminary data on the extent of infections of free-ranging marine turtles with the LETV and provide preliminary information about the impact LETV may have on marine turtle health. Since the Cayman Farm releases turtles on a regular basis and has no health monitoring program for herpesvirus infections (or other infections), this data will prove illuminating. In addition, The immunological data collected will provide critical seroepidemiological information for determining the relationship between infection with LETV and the subsequent development of LETD. This evidence may be used to strengthen the argument that LETV is the etiologic agent of LETD until transmission studies can be performed.

FPHV

Antigenic FPHV expressed peptides will be used to develop an FPHV ELISA following methods previously established for the LETV specific ELISA assay. The expressed FPHV peptides will be used as antigen to coat 96 well ELISA plates. Plasma samples previously tested by immunohistochemistry for presence or absence of anti-FPHV antibodies will be used to validate the

specificity of the assay. Plasma samples from captive-reared green turtles with no known exposure to herpesviruses (no anti-FPHV antibodies) will serve as the known-negative samples. Plasma samples from captive reared green turtles with experimentally induced FP (have anti-FPHV antibodies) will be used as known-positive samples. In addition, there is a set of plasma samples collected from wild green turtles with and without FP that have been previously shown to have plasma with and without anti-FPHV antibodies, respectively. These are important to obtain information about antibody levels in a naturally infected turtle. We will also utilize our unique monoclonal antibody reagents against marine turtle IgM, 7S IgG, and 5.7S IgG immunoglobulin subclasses as secondary antibodies so that early antibody responses (IgM) can be differentiated from late antibody responses (5.7S IgG).

Using the serological tests, such as the FPHV-specific ELISA, we will utilize a large plasma bank developed collaboratively with the University of Central Florida to identify marine turtles in Florida exposed to FPHV. This plasma bank contains samples from green and loggerhead turtles collect from the three study sites, Indian River Lagoon, Sebastian Inlet, and Trident basin (Indian River and Brevard Counties, FL), for over a decade. Unique serial (annual) samples from green turtles that were initially tumor free but developed tumors in subsequent years as well as samples from tumor regressor and progressor turtles are included in this plasma bank. The immunological data obtained by testing plasma from these various populations will be evaluated and will provide critical seroepidemiological information for determining the relationship between infection with the FP-associated herpesvirus and the subsequent development of fibropapillomatosis. This evidence may be used in lieu of or in association with transmission studies to strengthen the argument that the FP-associated herpesvirus is the etiologic agent of the disease. Transmission studies, the "gold standard" for fulfilling Koch's postulates must await cultivation of the virus.

10. OBJECTIVES OF PROJECT (maximum 4000 characters):

To express LETV antigens, evaluate wild marine turtle populations for exposure to LETV, and conduct seroepidemiological studies as follows:

a) PCR amplify open reading frames from genes that encode antigenic peptides of LETV identified from the genetic library, express the peptides, and screen for antigenicity.

b) PCR amplify regions of LETV genome not included in the shotgun library based on sequence information of neighboring genes to test additional gene products for antigenicity.

a) Identify peptides in LETV infected cells recognized by antibodies in LETV immunized plasma and wild green turtle plasma in the Western format.

a) Develop serological tests using expressed LETV peptides, and validate the assay with plasma samples previously screened for presence and absence of anti-LETV antibodies.

a) Screen various populations for exposure to LETV and provide evidence for exposure of wild marine turtles as well as critical seroepidemiological information for determining the relationship between infection with LETV and the subsequent development of LETD.

To express FPHV antigens and conduct seroepidemiological studies as follows:

a) PCR amplify open reading frames from genes that encode antigenic peptides of FPHV identified from the LETV genetic library, express the peptides, and screen for antigenicity.

b) Develop serological tests (like the ELISA developed for LETV) using expressed FPHV peptides, and validate the assay with plasma samples previously screened for the presence and absence of anti-FPHV antibodies.

a) Screen various populations for exposure to FPHV and provide critical seroepidemiological information for determining the relationship between infection with the FP-associated herpesvirus and the subsequent development of fibropapillomatosis.

13. PROGRESS STATEMENT (see complete report attached)(maximum 4000 characters):

Herpesviruses are associated with several diseases of marine turtles including lung-eye-tracheal disease (LETD) and fibropapillomatosis (FP). Critically needed are diagnostic tests for monitoring exposure of marine turtle populations to these herpesviruses. Using virus-infected cell lysates, we have developed and applied an ELISA to demonstrate that wild green turtles in Florida are exposed to the LETD-associated herpesvirus (LETV). In contrast, all attempts to cultivate the FP-associated herpesvirus (FPHV) have been unsuccessful, limiting diagnostic assay development and seroepidemiological studies. 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. 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. 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. Furthermore, prior to this study, genomic sequence information for marine turtle herpesviruses was limited. The only published genomic sequence information was for herpesviral DNA polymerase genes. To our knowledge, the antigenic proteins identified in this study are not only the first proteins from a reptilian herpesvirus to be cloned and expressed, but they represent the first reptilian herpesvirus proteins to be identified as immunogenic in their host species. In addition, these studies have approached the difficult topic of how marine turtle herpesvirus may be transmitted in a pilot experiment on vertical transmission in nesting turtles (manuscript in preparation). Finally, we have demonstrated using state-of-the art technology that a field portable assay for measuring exposure of chelonians to infectious agents is feasible. Taken together, 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. This work does move us closer to further understanding of herpesvirus infections in sea turtles and the development of assays to detect exposure of wild sea turtles to FPHV and other infectious agents that threaten their survival.

12. PROJECT SUMMARY STATEMENT (one or two hardhitting sentences that capture project merits):

We have developed immunological tests that can identify marine turtles in Florida (green and loggerhead) that have been exposed to the LETV herpesvirus. The seroepidemiological data collected provides critical evidence about the relationship between infection with the FP-associated herpesvirus and the LETV herpesvirus. The data supports the hypothesis that LETV and FPHV infections are independent infections of marine turtles. The data shows that wild green turtles in Florida are exposed to the LETD-associated herpesvirus, which is the first description of LETV infection in free-ranging marine turtles. To our knowledge, the antigenic proteins identified in this study are not only the first proteins from a reptilian herpesvirus to be cloned and expressed, but they represent the first reptilian herpesvirus proteins to be identified as immunogenic in their host species.

13.KEYWORDS (at least 1; up to 8): Fibropapillomatosis, marine turtles, lung-eye-trachea disease, herpesviruses, seroepidemiology, recombinant-viral antigens, ELISA,.

14. PUBLICATIONS (Cite all publications resulting from project including proceedings and technical reports. Use Journal of Wildlife Management style. Also, submit 5 reprints):

Coberley, S.S., Condit, R.C., Herbst, L.H., and **P. A. Klein**. Identification and Expression of Immunogenic Proteins of a Disease-associated Marine Turtle Herpesvirus. J. Virol. 76:10553-10558, 2002.

Coberley, S.S., Herbst, L.H, 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 47, 159-167, 2001.

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.

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.

15. PRESENTATION CITATIONS (Use Journal of Wildlife Management style, to include Name, Year, Title, Meeting/Conference Name, City and State.

Paul A. Klein, Daniel Brown, Elliott Jacobson, Lori Wendland, and Mary Brown. Microbial Pathogens, Immunology and Species Conservation. Workshop on Desert Tortoise Health and Disease. Soda Springs, California, November 14-17, 2002

Brown,M.B., **Klein , P.A.**, and Wendland, L.. Concepts and Importance of Disinfection. 28th Annual Meeting and Symposium of the Desert Tortoise Council, Las Vegas, NV, February 21-23, 2003

Coberley, S., Condit, R., Herbst, L., and **P. A. Klein**. The Development of Recombinant Viral Antigens for Detecting Herpesvirus Infections in Sea Turtles. *In* Proceedings in the Twenty-second Annual Symposium on Sea Turtle Biology and Conservation. Miami, Florida. April 4-7, 2002.

Coberley, S.S., Condit, R.C., Herbst, L.H., and **P. A. Klein** . Identification and Expression of Immunogenic Herpesviral for Detecting Herpesvirus Infections in Sea Turtles. American Society for Microbiology Annual Meeting, May 19th-23, 2002

Hirschmann, R. J., **Klein, P.A.**, Herbst, L.H., Ehrhart, L.M.,and Parkinson, C.L.
An investigation of vertical transmission in the role of the fibropapillomatosis-associated herpesvirus in marine turtles. Florida Keys Sea Turtle Symposium, Marathon, FL., December 6-7th, 2002

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 Biolog 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.

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.

16. THESES/DISSERTATIONS CITATIONS (Use Journal of Wildlife Management style. Also, submit 2 copies):

17. EMPLOYMENT STATUS OF GRADUATED STUDENTS (Provide Position Title, Agency/Company Name, City and State of employment of any graduated MS or PhD students who graduated):

18. HONORS/AWARDS:

19.

20. PERSONNEL:

NAME	POSITION*	GENDER	MINORITY**	DEGREE	PROGRAM***

Sadie Coberley, Graduate Student, F (UF), Ph.D.(2002), Interdisciplinary Program in Biomedical Sciences, COM,
Rachel Hirschman, Graduate Student, F, (UCF), M.S.(2003), Aquatic (Department of Biology, UCF)

Dean Bagley, Graduate Student, F, (UCF), M.S.,(2003), Aquatic (Department of Biology, UCF)

- MS Grad student; PhD grad student; Post-doc; Biologist; Technician;....

** B=Black; H=Hispanic; I=American Indian; O=Other; A=Alien

*** Aquatic, Terrestrial, or Integrated

20. NEWS MEDIA INVOLVEMENT:

Final Report
Research Work Order #213
January 20, 2004

Project Title: Seroepidemiological Studies of Herpesvirus-associated Diseases of Marine Turtles: Fibropapillomatosis and Lung-Eye-Trachea Disease

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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 determining the etiology of FP, 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. These new findings also raise new concerns about the potential impact of infections by new herpesviruses, such as lung-eye-trachea-disease virus on populations of wild marine turtles, an area which has previously been unexplored by turtle biologists.

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 (FP). 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. We have developed the first ELISA that can detect exposure of marine turtles to a specific herpesvirus infection (LETV). 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.

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. The data clearly shows that that LETV and FPHV infections are independent infections of marine turtles. Furthermore, prior to this study, genomic sequence information for marine turtle herpesviruses was limited. The only published genomic sequence information was for herpesviral DNA polymerase genes. The antigenic proteins identified in this study are not only the first proteins from a reptilian herpesvirus to be cloned and expressed, but they represent the first reptilian herpesvirus proteins to be identified as immunogenic in their host species. In addition, these studies have approached the difficult topic of how marine turtle herpesvirus may be transmitted in a pilot experiment on vertical transmission in nesting turtles (manuscript in preparation). Finally, we have demonstrated using state-of-the art technology that a field portable assay for measuring exposure of chelonians to infectious agents is feasible. Taken together, 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. This work does move us closer to further

understanding of herpesvirus infections in sea turtles and the development of assays to detect exposure of wild sea turtles to FPHV and other infectious agents that threaten their survival.

RESULTS SUMMARIES

Note: Copies of papers published are attached These contain the full details of results, materials, and methods. Other manuscripts are in preparation.

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

Note: Certain aspects of this research were fundedin part by RWO 180/194 to P.A. Klein as well as this RWO.

Manuscript: Curry, S., Brown, D.R., Gaskin, J.M., Jacobson, E.R., Ehrhart, L.M., Blahak, S., Herbst, L.H., and P.A. Klein. Persistent infectivity of a green turtle disease-associated herpesvirus after exposure to seawater. J. Wildlife Diseases, 36, 792-797, 2000

Summary: 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. They provide a proof of concept that herpesviruses associated with FP are likely to be infectious in the marine environment.

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

Manuscript: 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, 2001, 3572-3577.

Summary: 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

Manuscript: Coberley, S.S., Herbst, L.H, 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 47:159-167, 2001

Summary: 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. Identification of immunodominant LETV herpesvirus antigens

Manuscript: Coberley, S.S., Condit, R.C., Herbst, L.H., and **P. A. Klein**. Identification and Expression of Immunogenic Proteins of a Disease-associated Marine Turtle Herpesvirus. J. Virol. 76:10553-10558, 2002.

Summary: Herpesviruses are associated with several diseases of marine turtles including lung-eye-trachea disease (LETD) and fibropapillomatosis (FP). Diagnostic tests are critically needed for monitoring exposure of marine turtle populations to these herpesviruses. An ELISA assay was previously developed and applied using virus-infected cell lysates as antigen to document exposure of wild green turtles in Florida to the LETD-associated herpesvirus (LETV). In contrast, diagnostic assay development and seroepidemiological studies of FP have been limited by an inability to cultivate the FP-associated herpesvirus (FPHV). Antibodies to FPHV cross-react with LETV, suggesting a level of conservation of viral proteins between these two marine turtle herpesviruses. Expression of recombinant herpesviral proteins could provide an unlimited supply of antigen for LETV and FPHV diagnostic assays. However, herpesviral antigens recognized by the green turtle humoral immune response have not been identified. In this study, two approaches were used to identify immunodominant viral antigens of LETV. The first approach targeted viral proteins known to be immunogenic and neutralizing in other species and included glycoprotein B. The second strategy was to identify those immunodominant proteins recognized on Western blots by plasma antibodies from immunized or naturally infected green turtles. A 38 kDa protein was recognized by LETV and FPHV infected green turtle antibodies and was resolved by 2D gel electrophoresis. The protein was extracted, digested, and HPLC purified. The resulting protein fractions were sequenced and the protein was identified as a scaffolding protein encoded by the overlapping open reading frames of UL26 and UL26.5. Glycoprotein B and the scaffolding protein were PCR amplified based on the sequence of a partial LETV genomic library, and products were cloned and expressed in E. coli. The expressed proteins were recognized on Western blots by antibodies in immune green turtle plasma. These recombinant herpesviral proteins were evaluated extensively in ELISA assays as a source of antigen for screening marine turtle populations for exposure to herpesviruses. They were compared to the intact LETV herpesvirus which was used in all previous ELISA assays (see above). Unfortunately, these antigens could not reproducibly replace intact virions in the ELISA plate assays. The ELISA testing was exhaustive using nickel coated capture ELISA plates and recombinant antigens purified on nickel affinity columns and a wide variety of different types of ELISA plates, blocking buffers, and secondary antibodies. This result was not entirely surprising since these recombinant antigens were denatured during isolation due to the fact that they were not produced in a soluble form (were in bacterial inclusion bodies) by the bacterial hosts carrying their respective genes. Additional work is needed to develop new clones which secrete soluble forms of these immunogenic proteins for use in ELISA plate assays.

5. Investigation of Vertical Transmission in the Spread of Disease-Associated Herpesviruses in Marine Turtles at the Archie Carr National Wildlife Refuge, Florida

Manuscript in preparation: Hirschmann, R.J., P.A. Klein, L.M. Ehrhart, L.H. Herbst,and C.L. Parkinson1

Summary: Herpesviruses have been associated with several marine turtle diseases including fibropapillomatosis, lung-eye-trachea disease, and gray patch disease. Diseases can be transmitted horizontally from one animal to another by contact, or vertically, which is the spread of a disease from mother to offspring. The current study focused on the role that vertical transmission may play in the spread of the lung-eye-trachea disease-associated herpesvirus (LETV) and the fibropapillomatosis-associated herpesvirus (FPHV) in nesting marine turtles. Previous research has shown evidence of vertical transmission of herpesviruses in a large number of vertebrates, including humans, birds, fish, and amphibians. To determine if vertical transmission occurs in marine turtles, DNA was extracted from eggs laid by the nesting turtles, from the tissues of dead hatchlings, and from oviductal fluids secreted by the nesting turtles. A PCR (polymerase chain reaction) for the herpesvirus POL gene was used to indicate the presence of herpesvirus. Sequencing of all products was utilized to identify specific herpesviruses. These studies shed light upon the possible transmission mechanisms of marine turtle herpesviruses and provide valuable conservation information.

6. Development of Field Portable Chelonian Serodiagnostics

Manuscript in preparation: Daniel R. Brown, Marianne F. Kramer, Laurie A. Zacher, April M. Green, and Paul A. Klein Chelonian Serodiagnostics: Development of a Field Portable
Assay For Detection of Exposure of Tortoises to Mycoplasma agassizi.

[This study was conducted using tortoises and mycoplasma antigen but is currently being adapted for use in detecting herpesvirus ionfections of marine turtles.]

Summary: Infectious disease has affected plans for management and conservation of legally protected chelonians in the United States. Tortoise conservation and recovery plans now formally include testing for mycoplasmal Upper Respiratory Disease (URTD). Detection of specific anti-mycoplasma antibodies may be used to diagnose infection and immune status of chelonians as a tool for disease management. We have evaluated the feasibility of a field test for specific antibodies against mycoplasma in chelonian plasma, which would provide nearly instant information for management decision making. Preliminary trials were conducted of evanescent-wave biosensor technology for detection of specific anti-*Mycoplasma agassizii* antibodies in plasma from *Gopherus agassizii* tortoises. The evanescent-wave biosensor is a laser-based polystyrene fiber optic sensor which detects specific *G. agassizii* anti-*M. agassizii* antibody bound to *M. agassizii* whole-cell lysate antigen. The reporter molecule was Cy5-labeled HL637 monoclonal antibody against tortoise immunoglobulin. Under various experimental protocols, the signals from positive control plasma samples from our bank were three to seven times higher than the signals from negative control plasma samples. A randomized double-blind study was then conducted to determine the sensitivity, specificity, positive predictive value, and negative predictive value of the technique. Preliminary analyses of the results indicate a greater than 90% concordance with the traditional ELISA sample categorization, with a 5 minute per sample, field-portable protocol. Those results suggest that this technology is feasible for application under field conditions. Understanding the dynamics of disease spread in natural wildlife populations may also provide valuable new insights into host:pathogen:population interactions in this era of emerging infectious diseases.

The RAPTOR™ (*Research International, Woodinville, WA, 98072*) is a portable, 4-channel fluorometric assay system that can be used for high-sensitivity monitoring of biological agents, toxins, and other analytes. It represents a careful integration of optics,

fluidics, electronics, and software into one compact and rugged system for use in laboratory settings and field assays. This unit can automatically perform a user-defined, multi-step, assay protocol while simultaneously tracking fluorescently-tagged chemical reactions occurring on the surface of each of the system's four disposable optical waveguide sensors.

The RAPTOR™ portable, 4-channel fluorometric instrument.

Detection of antibodies to chelonian pathogens using the Raptor. The waveguide coated with herpesvirus antigen is first exposed to sea turtle plasma/blood and then to the CY-5 labeled anti-sea turtle IgG antibody. Laser light excites the bound CY-5 labeled antibody resulting in emitted fluorescence which is recorded. To run an assay, the user inserts a coupon into the RAPTOR, introduces the blood sample for testing, and presses the Run Assay key. All of the fluidics are contained within the instrument and are automated using computer-controlled miniature valves. The assay is complete in 15 minutes. The results are stored and can be displayed on the LCD or transferred to a computer. These assays are under further development.

DISCUSSION

All species of marine turtles have suffered serious population declines from over-harvesting for their eggs, meat, and shells; entrapment by fishing lines and nets; collisions with boats; dredging operations; and from destruction of nesting beaches and foraging habitat, and are currently either threatened or endangered. Permission to study or

conduct experiments with these animals is restricted due their endangered or threatened status. In addition, access to all life stages of turtles are further limited by their complex life cycle that takes an individual turtle over thousands of miles of pelagic ocean. Marine turtles traverse numerous marine habitats during their life history resulting in fragmented knowledge about marine turtle behavior and ecology at these various sites. Limited access has made it difficult to determine the impact of infectious diseases on marine turtle populations, even though it is well-established that infections with pathogens are capable of causing significant mortality in marine.

Worldwide experience has pointed to the central role of the immunological defense systems of all animal species in resistance to and recovery from infectious diseases. This is undoubtedly true in the case of marine turtles. Future improvements in the diagnosis and control of infectious diseases in marine turtle populations will require an increased understanding of the immunology of marine turtles, their associated pathogens, and the environmental factors which may undermine the immune system's ability to cope effectively with pathogens. Future research in this area will benefit from the networking of wildlife disease experts, immunologists, and biotechnology researchers to develop and utilize new tools and assays for management of diseases. Tools which are needed include up-to-date medical, genetic, and immunological databases, a wider variety of monoclonal antibodies to immune system components and pathogen-specific antigens, natural and recombinant pathogen-specific antigens for use in serological assays and as vaccines, and practical diagnostic immunoassays for measuring disease exposure and immune system function.

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 explored the development of viral and pathogen-specific recombinant antigens of both LETV and FPHV for use in seroepidemiological studies of these two diseases of marine turtles.

The ability to grow LETV in culture facilitated the development of an ELISA to assess exposure of populations of wild turtles to this herpesvirus. This has not been the case with the FP-associated herpesvirus to date. In this study, the LETV-specific ELISA successfully detected antibodies to LETV in plasma samples collected from 329 wild green turtles at three study sites on the east coast of Florida (estimated seroprevalence of 21.6%), the first report of this infection of these populations with this virus. Unfortunately, the developed recombinant immunodominant LETV antigens could not reproducibly replace intact LETV virions in ELISA plate assays. This result was not entirely surprising since these recombinant antigens were denatured during isolation due to the fact that they were not produced in a soluble form (were in bacterial inclusion bodies) by the bacterial hosts carrying their respective genes. Additional work is needed to develop new clones which secrete soluble forms of these immunogenic proteins for use in ELISA plate assays.

The data presented supports the hypothesis that LETV and FPHV infections of green and loggerhead turtles are independent. Taken together, 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 also raise important 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. This work moves us closer to further understanding of herpesvirus infections in marine turtles and the development of assays to detect exposure of marine turtles to FPHV and other infectious agents that threaten their survival.

PRESENTATIONS AND PUBLICATIONS FROM THIS PROJECT

Presentations.

Paul A. Klein, Daniel Brown, Elliott Jacobson, Lori Wendland, and Mary Brown. Microbial Pathogens, Immunology and Species Conservation. Workshop on Desert Tortoise Health and Disease. Soda Springs, California, November 14-17, 2002

Brown,M.B., **Klein , P.A.**, and Wendland, L.. Concepts and Importance of Disinfection. 28th Annual Meeting and Symposium of the Desert Tortoise Council, Las Vegas, NV, February 21-23, 2003

Coberley, S., Condit, R., Herbst, L., and **P. A. Klein**. The Development of Recombinant Viral Antigens for Detecting Herpesvirus Infections in Sea Turtles. *In* Proceedings in the Twenty-second Annual Symposium on Sea Turtle Biology and Conservation. Miami, Florida. April 4-7, 2002.

Coberley, S.S., Condit, R.C., Herbst, L.H., and **P. A. Klein** . Identification and Expression of Immunogenic Herpesviral for Detecting Herpesvirus Infections in Sea Turtles. American Society for Microbiology Annual Meeting, May 19th-23, 2002

Hirschmann, R. J., **Klein, P.A.**, Herbst, L.H., Ehrhart, L.M.,and Parkinson, C.L. An investigation of vertical transmission in the role of the fibropapillomatosis-associated herpesvirus in marine turtles. Florida Keys Sea Turtle Symposium, Marathon, FL., December 6-7th, 2002

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.

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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.

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Coberley, S.S., Herbst, L.H, 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 47, 159-167, 2001.

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.

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.

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assays to quantify plasma vitellogenin levels in green turtles (Chelonia mydas) Comp Biochem Physiol B Biochem Mol Biol, 135:551-63, 2003.

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F.C. Origgi, C. H. Romero, **P.A. Klein**, K. H. Berry, and E. R. Jacobson Serological and Molecular Evidences of Herpesvirus Exposure in Desert Tortoises from the Mojave and Colorado Deserts of California. Workshop on Desert Tortoise Health and Disease. Soda Springs, California, November 14-17, 2002

Origgi FC, Romero CH, **Klein PA**, Berry KH, Johnson A, and Jacobson ER Preliminary serological and molecular evidences of Tortoise Herpesvirus exposure in Desert Tortoises (*Gopherus agassizii*) from the Mojave and the
Colorado Desert of California. ARAV 8th Annual Conference, Reno, Nevada, October 17-21, 2002.