AN INVESTIGATION OF VERTICAL TRANSMISSION IN THE SPREAD OF DISEASE-ASSOCIATED HERPESVIRUSES IN MARINE TURTLES

by

RACHEL J. HIRSCHMANN
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ABSTRACT

Herpesviruses are associated with diseases afflicting marine turtles. I investigated the role of vertical transmission in the spread of two separate marine turtle herpesviruses. Vertical transmission of the fibropapillomatosis disease-associated herpesvirus (FPHV) was investigated in nesting green turtles (*Chelonia mydas*). Sixty-seven DNA samples, isolated from egg membranes and internal organs of hatchlings from fibropapillomatosis (FP) positive nesting turtles were screened for the DNA polymerase gene (POL) as a positive indicator for the presence of herpesviral DNA. Results indicate an absence of evidence for vertical transmission of FPHV or any herpesvirus. Vertical transmission of the lung-eye-trachea disease-associated herpesvirus (LETV) was also examined in nesting green and loggerhead (*Caretta caretta*) turtles. Plasma samples from 15 nesting loggerheads and 2 green turtles were tested for anti-LETV antibody levels using ELISA (enzyme-linked immunosorbent assay). DNA was isolated from oviductal fluid, fetus, and egg samples (n=33 total samples) originating from turtles with high anti-LETV levels. These samples were screened for the presence of the herpesviral DNA POL gene. Results indicate the presence of high anti-LETV levels in both loggerhead and green turtles; however, there was no evidence of vertical transmission of LETV or any herpesvirus. The results of these studies help to elucidate the modes of transmission of disease in marine turtles.

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LIST OF ABBREVIATIONS

ACNWR	Archie Carr National Wildlife Refuge
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CI	Chloroform/isoamyl alcohol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenedinitro tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FP	Fibropapillomatosis
FPHV	Fibropapillomatosis-associated herpesvirus
gB	Glycoprotein B
GTHV	Green turtle herpesvirus
kbp	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodalton
LETD	Lung-eye-trachea disease
LETV	Lung-eye-trachea disease-associated herpesvirus
M	Mole
mg	Milligram
mM	Millimole
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
NH_4	Ammonium
PCI	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase chain reaction
pmol	Picomole
POL	DNA polymerase gene
SLS	Sample loading solution
µl	Microliter
µM	Micromole
VZV	Varicella-Zoster Virus

CHAPTER 1: INTRODUCTION

All species of marine turtles that inhabit U.S. waters are listed as either endangered or threatened under the 1973 Endangered Species Act. These populations of marine turtles are imperiled by many factors including predation on eggs and hatchlings, human activity such as shrimp trawling and dredging, plus the degradation of foraging and nesting habitats (National Research Council 1990). Additionally, disease is a major threat to populations in the Atlantic Ocean (NMFS & USFWS 1991) and around the world.

Diseases impact marine fauna worldwide. The major etiological agents of diseases in marine animals include bacteria, fungi, metazoans, protozoans, and most relevant to this research, viruses. Viruses are obligate intracellular parasites that can multiply only within the living cells of their hosts. Once the virus has entered the host cell, the virus alters the host's metabolic processes and replicates the viral genetic material (Kinne 1980), which can be in the form of either DNA or RNA (Harper 1998).

The present studies focus on viruses belonging to the family Herpesviridae. Herpesviruses contain a double stranded DNA genome surrounded by protein to form an inner core. This inner core is enclosed within a capsid of icosahedral symmetry to make up a nucleocapsid approximately 100 nm in size. The mature virion is about 150-250 nm in diameter and is enveloped in a proteinaceous membrane (Fields et al. 1996). Replication and assembly of herpesviruses occurs in the nucleus where DNA that has infected the cell circularizes and replicates by a rolling circle mechanism. The viral DNA

progeny enter preformed capsids and exit the nucleus by budding. Transmembrane envelope proteins assemble to the nucleocapsids during budding. These mature viruses then transit through the Golgi network, and leave the cell by exocytosis (Ackermann et al. 1998).

Herpesviruses afflict a wide range of hosts, including birds, fish, mammals, and reptiles (Fields et al. 1996). Herpesviruses have been identified in the venom of two species of snakes, the banded krait (*Bungarus fasciatus)* and the Indian cobra (*Naja naja*; Wolf 1973), and have been isolated from green iguanas (*Iguana iguana*; Clark & Karzon 1972). The etiological agents of diseases in catfish and salmon are herpesviruses, the channel catfish virus (Wolf 1973), and herpesvirus salmonis, respectively (Wolf 1975). Fibropapillomatosis (FP), lung-eye-trachea disease (LETD), and gray patch disease, have been associated with herpesviruses and affect marine turtles (Rebell et al. 1975, Jacobson et al. 1986, Jacobson et al. 1991).

Spread of herpesviruses can occur by two mechanisms: horizontal and vertical transmission. Horizontal transmission involves the spread of a disease from one animal to another by contact. For example, ovine herpesvirus-2 (OvHV-2), the causative agent of sheep-associated malignant catarrhal fever, was transmitted by contact from sheep to Japanese deer (*Cervus nippon*) after the two animals were kept in close quarters with one another (Imai et al. 2001). Vertical transmission is the spread of the pathogen from one generation to another, for example, the passage of a virus from a mother to her offspring. The mechanisms through which vertical transmission may occur include the transfer of

virus via infected germ-line cells (ova or sperm), or through viral shedding in the reproductive tract that would produce viral infection in the ovaries.

Vertical transmission of herpesviruses has been documented in many vertebrates. The Kaposi's sarcoma-associated herpesvirus (KSHV) in humans is thought to be spread to children via perinatal transmission or breast-feeding (Olsen & Moore 1998). Studies performed on birds have shown vertical transmission of herpesvirus from the mother to the eggs (Burgess & Yuill 1980). The herpesvirus infection associated with duck plague was shown to be vertically transmitted from the mother to the egg in muscovy (*Cairina moschata*), pekin (*Anas platyrhynchos domesticus*), and mallard (*Anas platyrhynchos)* duck species (Burgess & Yuill 1980). Herpesvirus salmonis has been isolated from ovarian fluids of salmonids, including the rainbow trout (*Oncorhynchus mykiss*) and landlocked sockeye salmon (*Oncorhynchus nerka;* Wolf 1975). Circumstantial evidence supports the hypothesis that the channel catfish virus is vertically transmitted to eggs (Wolf 1973, Davison 1992). Previous works on amphibians and reptiles, including frogs and iguanas, have shown evidence of vertical transmission of herpesviruses. Experimental transmission of Ranid herpesvirus 1 (RaHV-1), the etiological agent of Lucke renal adenocarcinoma in northern leopard frogs (*Rana pipiens*), provided evidence for vertical transmission (McKinnell 1973, Davison et al. 1999). It has also been hypothesized that the Iguana virus may be transmitted vertically in green iguanas (Wolf 1973).

Previous studies on vertical transmission have used various molecular techniques, including polymerase chain reaction (PCR) and Southern blot hybridization to determine

the presence or absence of viral DNA in parents and offspring. While these studies have shown vertical transmission of herpesviruses in a broad range of vertebrates, transmission of viruses in marine turtles has yet to be investigated. I used PCR as a tool to detect presence of viral DNA in the offspring of diseased nesting turtles. The genomes of both the lung-eye-trachea disease-associated herpesvirus (LETV) and the fibropapillomatosis-associated herpesvirus (FPHV) have yet to be sequenced, however, at least one gene has been well characterized. The DNA polymerase gene (POL) of FPHV has been identified and sequenced in numerous FP positive turtles. This gene is responsible for the replication of genomic information (Knopf 1998), and seems highly conserved between isolates from Florida and Hawaii, as determined by sequence alignment (Quackenbush et al. 1998). I used primers that amplify a 483 bp portion of the DNA POL gene of FPHV in this study. PCR primers which amplify a 224 bp region of the DNA POL gene of the lung-eye-trachea disease-associated herpesvirus (LETV) were also used in the present studies, as this gene is the only one for which primers have previously been developed.

The present studies investigated the role of vertical transmission in the spread of disease-associated herpesviruses in green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) turtles at the Archie Carr National Wildlife Refuge (ACNWR), Florida. The first study tested the hypothesis that the FPHV connected with FP disease is vertically transmitted in nesting green turtles. Using PCR primers for the FPHV DNA POL gene, I systematically tested for the presence of this gene in the offspring (eggs and internal organs of hatchlings) of FP positive females, which would indicate the presence of the FPHV genome in the offspring. Such evidence would provide support for the hypothesis

that offspring could contract FP from their mothers. The second study tested the hypothesis that the LETV connected with the LETD is vertically transmitted in nesting green and loggerhead turtles. Plasma from nesting turtles was tested for anti-LETV antibodies by using ELISA (enzyme-linked immunosorbent assay). Oviductal fluids and eggs from turtles with high anti-LETV levels were tested for the presence of the LETV DNA POL gene using PCR primers for this gene. Degenerate PCR primers that amplify most herpesviral DNA POL genes were also used in both the FPHV and LETV transmission studies.

CHAPTER 2: VERTICAL TRANSMISSION OF FIBROPAPILLOMATOSIS

Introduction

Fibropapillomatosis is a debilitating and often fatal neoplastic disease afflicting marine turtles. FP is characterized by benign cutaneous fibropapilloma masses ranging from 0.1 cm to greater than 30 cm in diameter that can be smooth or rough in texture, and some even appear stalk like. These tumors are commonly found on the conjunctiva, chin, neck, flippers, inguinal areas, axillary areas, and the tail base. Visceral nodules are frequently found in the lungs, kidneys, heart, liver, and gastrointestinal tract (Herbst 1994).

Fibropapillomatosis was first described by Smith and Coates (1938) in a captive green turtle (*Chelonia mydas*) at the New York Aquarium. This disease has now been documented in flatback (*Natator depressus*), loggerhead (*Caretta caretta*), and olive ridley (*Lepidochelys olivacea*) turtles (Herbst et al. 1998, Quackenbush et al. 1998, Lackovich et al. 1999). However, FP occurs at highest frequencies in large juveniles and adult green turtle populations (Herbst 1994). Fibropapillomatosis is found in marine turtles worldwide, but the highest prevalence of the disease is in Hawaii (Work et al. 2000) and in Florida (Herbst 1994). Lagoons and near-shore bays with low flushing rates and local development have higher incidences of FP afflicted turtles than other marine habitats (Herbst 1994).

The etiological agent of FP is not known, but an infectious viral agent is strongly implicated (Jacobson et al. 1991, Herbst et al. 1994, Herbst et al. 1995, Herbst et al. 1996,

Quackenbush et al. 1998, Lackovich et al. 1999). Using electron microscopy, Jacobson et al. (1991) reported particles similar to viruses associated with the family Herpesviridae in cutaneous and ocular fibropapillomas of two juvenile green turtles. Herbst et al. (1995) experimentally transmitted FP to green turtles using filtered cell-free tumor homogenates, which further implicated a viral etiology, and demonstrated herpesviral particles in donor and recipient tumors (Herbst et al. 1995, Herbst et al. 1996, Herbst et al. 1998). Lackovich et al. (1999), using nested consensus-primer PCR methodology, detected the presence of the herpesviral DNA polymerase I gene (POL) in 96% of all tumors tested from FP positive green and loggerhead turtles. Lackovich et al. (1999) also detected herpesvirus in 9% of skin samples of FP positive turtles; all samples that contained the herpesvirus were within 2 cm of a tumor. POL was partially sequenced from FP-associated herpesvirus (FPHV) in tumor tissue (Herbst et al. 1998, Quackenbush et al. 1998, Lackovich et al. 1999). Sequence data of the FPHV DNA POL gene indicate that it is a member of the *Alphaherpesvirinae* subfamily (Quackenbush et al. 1998, Yu et al. 2000). Additional PCR studies using FPHV specific primers consistently detected FPHV DNA POL sequences in all tumor tissues, and in 80% of non-tumored tissues/organs of FP positive turtles (Lu et al. 2000). Viral sequences were not detected in any of the 28 tissues of non-tumored turtles (Lu et al. 2000). Despite evidence supporting the hypothesis that a herpesvirus is the etiological agent of FP, Koch's postulates have not been fulfilled since the herpesvirus associated with FP has not been isolated and cultured. Until this is done, the hypothesis that FPHV is the causative agent of FP will remain unproven.

Several aspects of the FP disease are unclear and require further elucidation to fully understand how this disease affects its hosts. A major question remaining is how FP is transmitted. A high density of diseased and or susceptible animals in an area has been suggested to enhance disease transmission (Herbst 1994), as illustrated in the horizontal transmission of damselfish neurofibromatosis (Schmale 1991). Such large aggregations of marine turtles occur at feeding grounds where density dependant transmission of diseases, such as FP, would take place. Curry et al. (2000) showed that the lung-eye-trachea disease-associated herpesvirus (LETV), a herpesvirus associated with marine turtles, can remain infectious in seawater for up to 5 days, supporting transmissibility in a marine environment. Vertical transmission, the spread of a pathogen from one generation to another, has been shown to occur in a suite of vertebrates, including amphibians, birds, fish, and humans. Vertical transmission of disease has yet to be investigated in marine turtles. Studying this aspect of transmission is integral to understanding the etiology of FP in marine turtles.

In this study I tested the hypothesis that the FPHV associated with FP is vertically transmitted in nesting green turtles with FP (All turtles originating from ACNWR, Florida). Eggs and hatchlings of FP positive turtles were dissected and total DNA was isolated from different egg membranes and hatchling organs. Using PCR primers for the FPHV DNA POL gene, I systematically tested for the presence of FPHV DNA in the offspring tissues. I also investigated the potential presence of any herpesviral DNA in the offspring by using degenerate PCR primers that amplify most herpesviral DNA POL genes.

Methods

Sample collection

Eggs and hatchlings were collected from seven FP positive nesting green turtles from May-August of 2000, 2001, and 2002 at the ACNWR, Florida. FP positive status of turtles was determined by the presence of external fibropapilloma tumors. These females were tagged, surveyed for morphometric data, and visible tumors were measured and described. The location of each nest was marked. Seventy days post-oviposition, unhatched eggs and dead hatchlings were collected for analysis using sterile plastic bags labeled with the following information: turtle tag numbers, date nest was laid, and date of inventory. To prevent cross-contamination, new latex gloves were used to inventory each nest. All eggs present were collected, both hatched and unhatched, and were placed onto a sieve. Hatched eggs were counted, and then placed back in the nest, while unhatched eggs and dead hatchlings were counted, deposited into a prelabeled bag and frozen.

DNA isolation

A total of 11 eggs and 13 hatchlings from 7 FP positive females and 8 nests were dissected. Internal egg membranes, egg shells, and the following tissues of dead hatchlings were collected for DNA isolation: kidney, heart, liver, lung and skin (n=67 individual tissue samples). As a positive control, DNA was isolated from tumor tissue from FP positive turtles. Tissue samples were digested in 500 µl Rosetti's Buffer (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 0.6% SDS, 25 mM EDTA pH 8.0), .2 mg

Proteinase K, and 10 mg trypsin for 48 hours at 37° C. After digestion, each sample was extracted 2 times with 500 µl phenol, vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and was extracted 2 times with 500 µl of PCI (50 phenol:48 chloroform:2 isoamyl alcohol), vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and was extracted 2 times with 500 µl CI (48 chloroform:2 isoamyl alcohol), vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and DNA was precipitated with 1/10 final volume 3M sodium acetate and 2 X final volume 100% ice cold EtOH. Samples were placed on ice for 60-120 minutes, washed with 70% ice cold EtOH, air dried, and resuspended in 200 µl 1X TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0). DNA samples were quantified on a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) and visualized on a 1.1% agarose gel (Fig 1).

Amplification and sequencing

An array of strategies were employed to screen turtle DNA samples, via PCR amplification, of the DNA POL gene of FPHV. Standard primer pair amplification (GTHV1/GTHV2; GTHV2/GTHV3) was used in addition to nested PCR using degenerate primers which amplify most herpesviruses (Table 1). Variations in PCR reaction chemistry were tested to identify optimal reaction ingredients for nested and standard PCR. A diversity of thermocycling parameters were also utilized to optimize

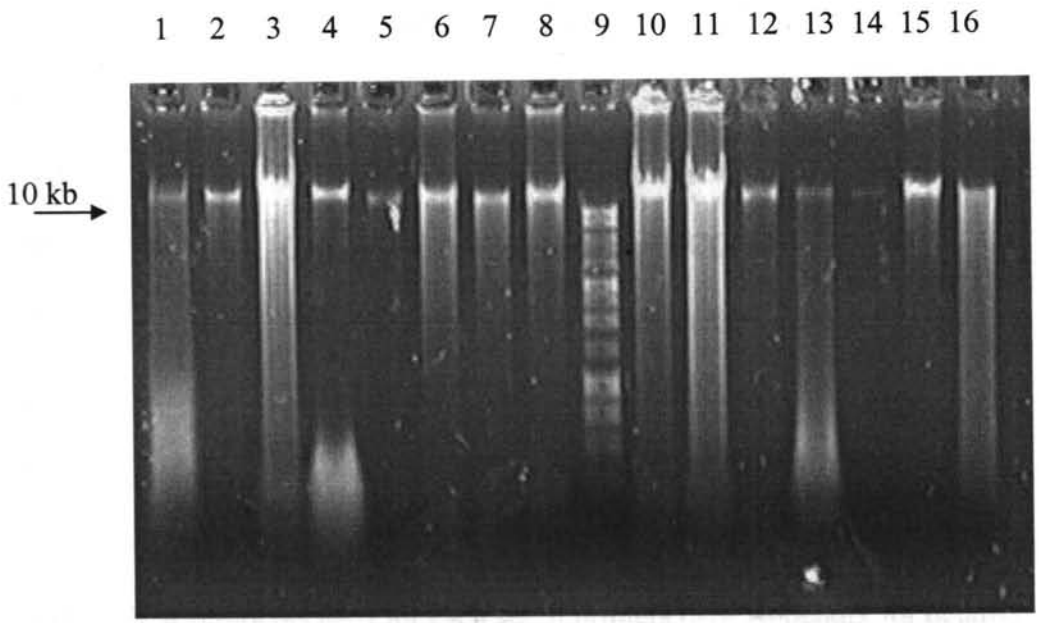

Figure 1. Products of DNA isolation from eggs and internal organs of hatchlings. Lane 1: (X8709) egg shell; Lane 2: (X8874) inner membrane of egg; Lane 3: (X8709) inner membrane of egg; Lane 4: (X8907) amnion; Lane 5: (X8907) inner membrane of egg; Lane 6: (CLP 156) kidney; Lane 7: (CLP 157) liver; Lane 8: (CLP 157) heart; Lane 9: Promega 1 kb ladder; Lane 10: (CLP 204) lung; Lane 11: (CLP 205) heart; Lane 12: (X8709) inner membrane of egg; Lane 13: (CLP 257) liver; Lane 14: (W2859) inner membrane of egg; Lane 15: (CLP 262) heart; Lane 16: (CLP 262) skin

Table 1. PCR primer sequences (5'-3'); Ambiguous codes follow IUB standards

Primer	Sequence
PAK1	GTCCTTGCTACCANTCNACNCCYTT
PAK2	TGTAACTCGGTGTAYGGNTTYACNGGNGT
PAK3	CACAGAGTCCGTRTCNCCRTADAT
PAK6	TCCTGGACAAGCRNYSGCNMTNAA
GTHV1	TGTCTGGAGGTGGCGGCCACG
GTHV2	GACACGCAGGCCAAAAAGCGA
GTHV3	AGCATCATCCAGGCCCACAA
CR1	TTGTACATCTACTTATTTACCAC
CR2	GTACGTACAAGTAAAACTACCGTATGCC

Table 2. PCR protocols used for each set of primers (See Appendix for details)

PCR primers	Protocol # used
PAK1/PAK6/PAK2/PAK3	1, 5, 6, 7
GTHV1/GTHV2	2, 3, 4, 8, 9
GTHV2/GTHV3	2
CR1/CR2	10

amplification conditions (Table 2, Appendix). All products resulting from PCR amplification were sequenced and identified using NCBI BLAST algorithm.

A range of DNA (1-800ng), dNTP (.3-.8mM), and magnesium chloride (1.5-4mM) concentrations, plus the inclusion and exclusion of 1% DMSO, were used in combination with the different cycling conditions (Appendix). Also, different brands of *Taq* polymerase were tested (Sigma, St. Louis, MO; and Bioline, Randolph, MA), each with different buffers (KCl and NH_4 respectively). A positive and a negative control (deionized water) were used in each round of PCR.

As an additional control to test DNA isolation procedures, PCR primers, amplification conditions, and reaction optimization, DNA was isolated from FP tumor and was used in a PCR reaction using GTHV2/GTHV3 primers. In each 25 µl reaction, .4 mM dNTPs, 1 µM each primer, 2 mM magnesium chloride, 2.5 µl Sigma buffer (St. Louis, MO), and 1.25 units Sigma *Taq* (St. Louis, MO) were used.

An initial set of nested PCR reactions using two sets of degenerate primers (PAK1/PAK6 and PAK2/PAK3) at a final concentration of .5 µM per primer was conducted (Fig 2). The primers (PAK2/PAK3) used in the second round of PCR amplify a 224 bp conserved region of the herpesviral DNA POL gene (VanDevanter et al. 1996). Varicella zoster virus (VZV) DNA (100 ng) was used as a positive control in these reactions. PCR was performed on all samples using specific primers (GTHV1/GTHV2; 1µM each primer), designed for a 165 bp region of the FPHV DNA POL gene (Quackenbush et al. 1998; Fig 2). Each sample was also tested with a second set of specific primers (GTHV2/GTHV3; 1µM each primer), which yields a 483 bp fragment of

the FPHV DNA POL gene (Quackenbush et al. 2001; Fig 2). FP tumor genomic DNA containing FPHV DNA, previously isolated from an FP tumor, was used as a positive control in all specific PCR reactions. I used 50 ng of positive control for the GTHV1/GTHV2 PCR, and 400 ng positive control for the GTHV2/GTHV3 PCR. Sizes of PCR products were determined on a 1.5% agarose gel with either AmpliSize (Bio-Rad, Hercules, CA) or pGEM (Promega, Madison, WI) DNA ladder and visualized using ethidium bromide staining.

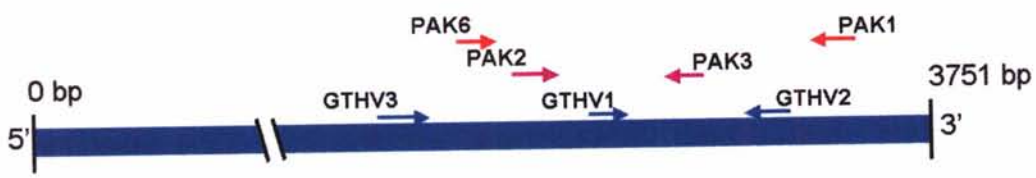

Figure 2. PCR primers used for the amplification of the herpesviral DNA POL gene

As an added positive control to assess the quality of isolated DNA, 6 DNA samples were used in PCR reactions using the primer pair CR1/CR2, which amplify a 400 bp region of the mitochondrial d-loop region of green turtles (Norman et al. 1994). In each 25 µl reaction, .04 mM dNTPs, .5 µM each primer, 1.5 mM magnesium chloride, 2.5 µl Sigma buffer (St. Louis, MO), .1 µl DMSO, and .5 units Sigma *Taq* (St. Louis, MO) were used.

Positive PCR products were gel purified and isolated using the Qiagen MinElute Gel Extraction Kit (Qiagen Inc., Valencia, CA). Purified PCR products from the FP tumor and mtDNA assays were quantified on a 1.2% agarose gel using Low Mass Ladder (Invitrogen, Carlsbad, CA). These products were directly sequenced using 2µM primers, 20 ng PCR product, and 2 µl Quick Start Ready Reaction Mix from the CEQ Dye-Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA). Cycling conditions were 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes, for a total of 34 cycles. Sequencing reactions were centrifuged at 4000 rpm for 20 seconds, and 2.5 µl of stop solution (1 µl 3M sodium acetate pH 5.2, 1 µl 100 mM EDTA pH 8.0, 0.5 µl 20 mg/ml glycogen) was added to each reaction. Sequencing reactions were precipitated with 60 µl ice cold 95% EtOH, sealed, inverted vigorously 5-10 times, and placed at –20° C for 10 minutes. The samples were then centrifuged at 4000 rpm for 10 minutes. The plate was inverted to remove the supernatant and centrifuged upside down at 300 rpm for 20 seconds. The pellets were rinsed 2 times with 200 µl ice cold 70% EtOH. Following each rinse, the plate was centrifuged at 4000 rpm for 3 minutes, inverted to remove the supernatant, and centrifuged upside down at 300 rpm for 20

seconds. The samples were then vacuum dried for 10 minutes, resuspended in 20 µl SLS buffer (Beckman-Coulter, Fullerton, CA) and separated on a Beckman CEQ2000 automated sequencer according to manufacturer's protocols. Raw sequence chromatographs were edited using Sequecher 4.1 (Gene Codes, Ann Arbor, MI), and sequence identity was inferred by searching the NCBI databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Purified PCR products other than controls (FP tumor and mtDNA PCR samples) were cloned to search for the possibility of various herpesviruses in PCR products. Cloning was completed using Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Multiple clones from a single PCR product were grown overnight in liquid culture (LB plus ampicillin). Plasmids with inserts were then purified using the QiaPREP Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Plasmids were sequenced using M13 primers with the CEQ Dye-Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA). Each reaction contained approximately 300 ng of plasmid DNA, 4 pmol of each primer, and 2 µl of Quick Start Ready Reaction Mix (Beckman-Coulter, Fullerton, CA). Samples were sequenced, edited, and identified as described above.

Results

One of 67 DNA samples produced a positive result using nested PCR under protocol 1 (Appendix) and PAK1/PAK6/PAK2/PAK3 primers. This sample originated from the inner membrane of an egg. BLAST results identified the sequence as being most similar to *Escherichia coli* 0157:H7 EDL933 genome (Score 86, E-value 4e-14).

The remaining 66 samples produced negative results (Fig. 3). Positive and negative controls worked for each PCR assay.

None of the DNA samples tested with the GTHV1/GTHV2 primers produced positive results. Each PCR assay had a working positive and negative control.

Thirty of 67 DNA samples (45%) produced appropriate sized bands using protocol 2 (Appendix) and GTHV2/GTHV3 primers (Fig 4). BLAST search indicated these sequences were homologous to the following bacterial genes: *Salmonella typhimurium* LT2 section 14 of 220 of complete genome, *Escherichia coli* CFT073 section 2 of 18 of complete genome, and *Shigella flexneri* 2a. strain 301 section 10 of 412 of complete genome.

Five of 6 DNA samples (inner membrane of 2 different eggs, kidney, heart, lung) produced positive results using protocol 10 (Appendix) and CR1/CR2 primers (Fig 5). BLAST results identified all sequences as homologous to *Chelonia mydas* mitochondrial d-loop (Score 751, E-value 0).

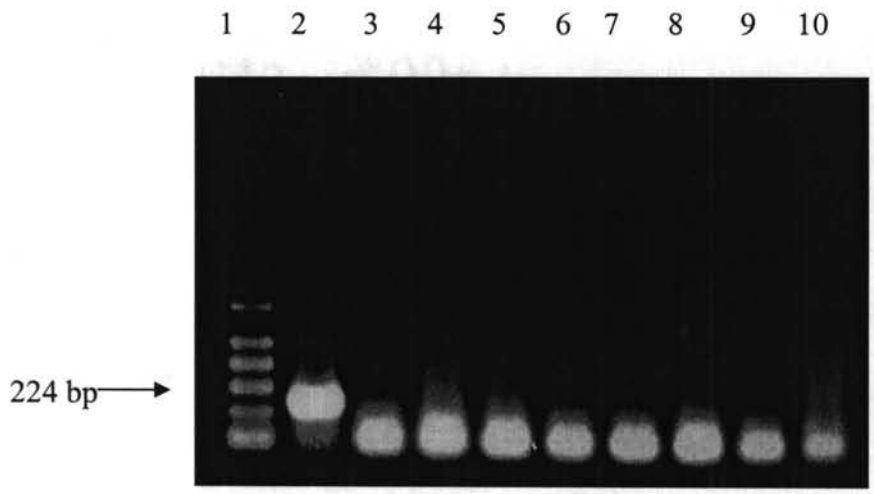

Figure 3. Absence of herpesviral DNA POL gene in eggs with PAK1/PAK6 and PAK2/PAK3 primers in a nested format. Lane 1: Amplisize marker; Lane 2: VZV DNA (positive control); Lane 3: deionized water (negative control); Lane 4: (X8709) shell; Lane 5: (X8709) shell; Lane 6: (X8709) inner membrane; Lane 7: (X8874) inner membrane; Lane 8: (X8874) shell; Lane 9: (X8907) inner membrane; Lane 10: (X9251) plastron tissue.

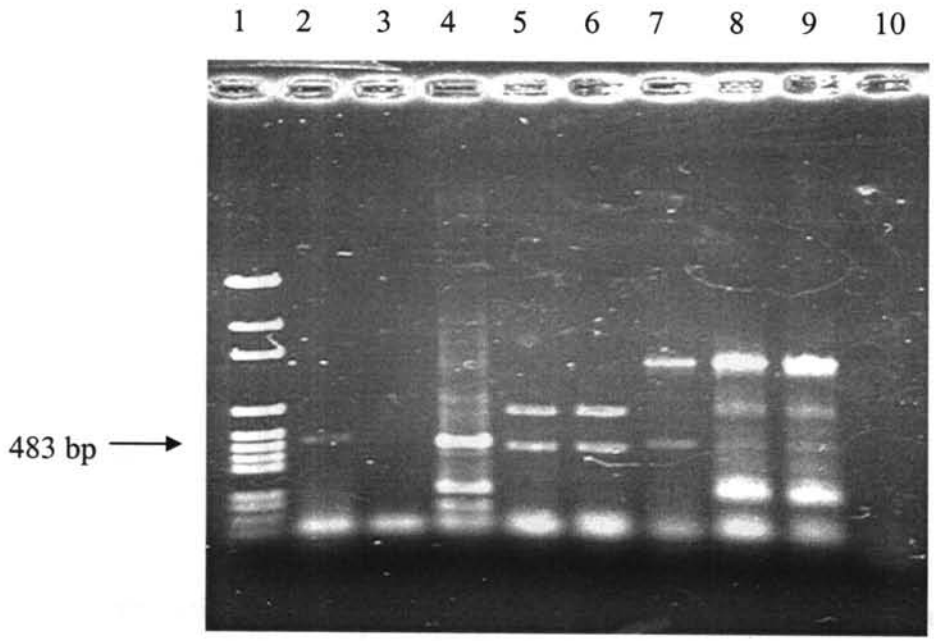

Figure 4. PCR amplification using GTHV2/GTHV3 primers. Lane 1: pGEM size marker; Lane 2: FPHV tumor DNA (positive control); Lane 3: Deionized water (negative control); Lane 4: (X8709) inner membrane; Lane 5: (X8874) inner membrane; Lane 6: (X8874) inner membrane and shell; Lane 7: (X8907) liver from fetus; Lane 8: (X8907) amnion; Lane 9: (X8907) amnion; Lane 10: (X9251) skin.

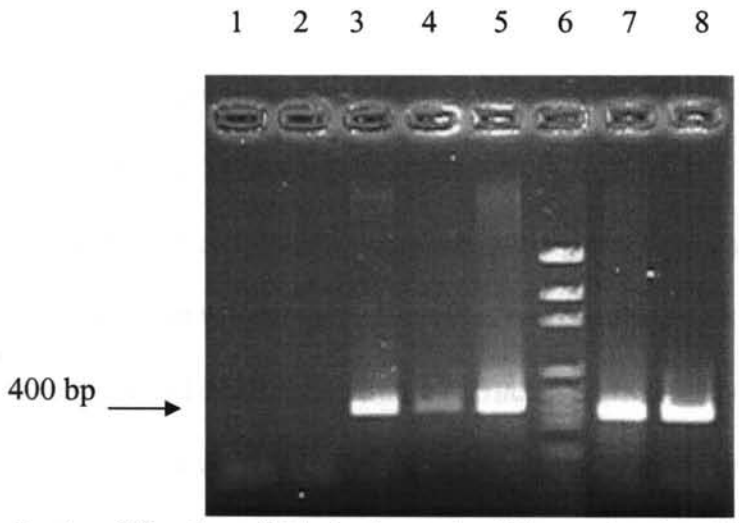

Figure 5. Amplification of *Chelonia mydas* d-loop mitochondrial gene in eggs and internal organs of hatchlings. Lane 1: Deionized water (negative control); Lane 2: (X8874) internal membrane of egg; Lane 3: (X8709) internal membrane of egg; Lane 4: (CLP 156) kidney; Lane 5: (CLP 205) heart; Lane 6: pGEM size marker; Lane 7: (CLP 257) lung; Lane 8: (W2859) internal membrane of egg.

Discussion

Herpesviruses afflict a wide range of hosts (Fields et al. 1996) and are transmitted vertically and horizontally. Studies of avian herpesviruses demonstrated these pathogens to be vertically transmitted from mothers to developing eggs (Burgess & Yuill 1980). Horizontal transmission, on the other hand, may occur at areas with high densities of animals; for example, at breeding grounds where animals may be in close contact with one another. However, these two routes of transmission are not mutually exclusive, and have the possibility of occurring simultaneously.

Many aspects of fibropapillomatosis pathogenesis are currently unknown, particularly modes of transmission. Previous work indicates a strong correlation between PCR detection of FPHV DNA and turtles afflicted with FP. Therefore, to look for evidence of vertical transmission of this virus, I hypothesized that FPHV DNA would be present in the offspring of diseased mothers. Using the DNA POL gene as a positive indicator for the presence of herpesviral DNA, including FPHV, no instances of a positive herpesviral DNA marker was amplified in the present study. The results of this study did not support the hypothesis that FPHV can be transmitted vertically.

Lu et al. (2000) was unable to detect FPHV sequences in tissues of turtles that did not exhibit signs of FP (non-tumored turtles); however, FPHV sequences were found in tumors and non-tumor tissues of turtles that exhibited signs of FP. The hatchlings and fetuses tested via PCR in these studies did not exhibit signs of tumor growth. If FPHV is the cause of FP, the animal must be infected with the herpesvirus before the disease can occur. If vertical transmission was occurring, we should have been able to detect viral

DNA in the offspring tested even though there were no visible tumors. The vertically transmitted virus can be expressed phenotypically later in life; until these tumors are visible, this herpesvirus may be latent (not active) in the turtle.

Although there is a strong association of FPHV with tumors of FP positive turtles, it is uncertain whether this herpesvirus is the primary cause of this disease. Herpesviruses have been noted to colonize tissues and tumors of injured animals; therefore, the presence of herpesviral DNA in the tumors may represent a secondary infection (Herbst 1994; Herbst et al. 1995; Herbst et al. 1999). Searching for the evidence of FPHV in the offspring of FP afflicted turtles (assuming it was the cause of the disease) would provide us with negative results if this herpesvirus is a secondary infection acquired later in life, and if this herpesvirus only infects some turtles with FP after they get sick, and therefore isn't transmitted.

The negative results of this study may be a result of an alternate route of transmission of FPHV. Horizontal transmission of FPHV has been proven experimentally (Herbst et al. 1995, Herbst et al. 1996). The absence of evidence for vertical transmission in this study indicates that horizontal transmission of FPHV in green turtles warrants further investigation. High densities of marine turtles in a single area, such as breeding or feeding grounds, may enhance disease transmission (Herbst & Klein 1995). There is a high prevalence of FP in turtles residing in near-shore bays and lagoons (common feeding habitats for marine turtles) where human activity is common (Herbst & Klein 1995). Herpesviruses can survive for extended periods of time in a marine environment, outside their hosts (Curry et al. 2000). Lagoons and near-shore bays tend to

have low-flushing rates, which may favor the build-up of infectious agents (Herbst & Klein 1995). Activities such as dredging and fishing increase stress on turtles, and therefore make them more susceptible to diseases, such as FP (Herbst & Klein 1995).

The absence of herpesviral DNA in the offspring tested may also be an effect of sample size. Viral DNA may be present in some of the eggs that were not tested. Sample size was limited to numbers of eggs left in the nest at inventory. It is also possible that parts of the eggs and or hatchlings that harbor the virus were not tested and therefore were not detected in the present study. Herpesviruses, as with most enveloped viruses, are sensitive to storage conditions (Herbst et al. 1995), so viral degeneration in the samples may appear to be a possible explanation of the negative results. However, sequencing of positive PCR products from the mitochondrial PCR assay indicated that they were homologous to the *Chelonia mydas* mitochondrial d-loop. These results confirm the quality of the templates used in the PCR assays and suggest that the DNA isolated from samples was not degraded. The DNA sample which produced a negative result showed to be of good quality based on results of an agarose gel following DNA isolation. Therefore, this sample may contain a PCR inhibitor.

Sequencing results indicated the presence of the Florida green turtle herpesviral DNA POL gene in all tumor samples tested. These results validate the successful isolation and sequencing of FPHV DNA POL; thereby indicating that if viral DNA was in the offspring of FP positive turtles, it should have been detected by the protocols employed in the present study.

Because this is a small study, the question of vertical transmission warrants further investigation; however, this mode of transmission would have to be a common event to be detected. Future studies may benefit from testing a larger sample of eggs from each nest, a larger number of nests, and more samples from each egg, as well as artificially incubating eggs of FP positive females. Techniques other than PCR, such as Southern blots, could be used to detect herpesviral DNA to determine if FPHV is vertically transmitted. Southern blots are less sensitive than PCR, but could be used to verify a positive PCR product. Wise et al. (1988) successfully used Southern blots to accurately detect the presence of a herpesvirus, the channel catfish virus, in the offspring of diseased catfish (*Ictalurus punctatus*). PCR is one of the most sensitive assays, and other studies using less sensitive techniques found vertical transmission, so we should have found it as well. If we had found a positive PCR product, we could use Southern blots to confirm it. Further sequencing of the FPHV DNA POL gene may allow researchers to design new PCR primers that may be able to better detect FPHV in the offspring of FP positive mothers. Future studies, at the molecular, immunological, and ecological levels are needed to better understand the pathogenesis of this debilitating disease. These data are paramount for better management of threatened and endangered species of marine turtles worldwide.

Future studies determining the commonality of vertical versus horizontal transmission of marine turtle herpesviruses will have important conservation implications. Vaccines have the potential to control disease by artificially inducing immunity (Bloom & Lambert 2003), so they may prove useful in preventing the spread of

the FP disease. If vertical transmission is proven to be more common relative to horizontal transmission, and it is shown that this transmission is occurring via viral shedding into the reproductive tract, then vaccinating diseased females may help control the spread of this disease. If it is proven that horizontal transmission is more common than vertical transmission, vaccinating both sexes of turtles at all life stages will be crucial to controlling FP, and ultimately aiding in the protection of marine turtle populations throughout the world.

CHAPTER 3: VERTICAL TRANSMISSION OF LUNG-EYE-TRACHEA DISEASE

Introduction

Herpesviruses are associated with several diseases in marine turtles. The lung-eye-trachea disease-associated herpesvirus is the only such virus of marine turtles to be isolated and propagated in culture (Jacobson et al. 1986, Coberley et al. 2002). LETV has not been completely identified as the etiological agent of LETD as transmission studies of LETD have not been conducted; however, the LETV was isolated from involved tissues. LETV is a stable virus and was shown to remain infectious after extended exposure to seawater (Curry et al. 2000).

The first epizootic occurrence of LETD was documented in 1975 in captive reared juvenile green turtles at the Cayman Turtle Farm, Grand Cayman, British West Indies (Jacobson et al. 1986). Characteristics of this disease included conjunctivitis, tracheitis, and pneumonia. Turtles afflicted with LETD exhibited buoyancy abnormalities, problems diving, and an increased tendency for respiratory gasping.

Only recently has there been serological evidence indicating LETV infection in wild turtles, in addition to captive raised turtles. Coberley et al. (2001a) developed an ELISA test for detection of anti-LETV antibodies in marine turtles, and showed the first evidence of wild green turtle exposure to LETV. This LETV specific ELISA was then applied to three populations of juvenile green turtles, and adult nesting green and loggerhead turtles on Florida's east-central coast (Coberley et al. 2001b). Twenty-one percent of all turtles sampled (71/329) were seropositive for LETV antibodies (Coberley

et al. 2001b), demonstrating previous exposure to LETV in free-ranging green turtles. Coberley et al. (2002) cloned and expressed two LETV proteins (gB and 38-kDa), and identified LETV as a member of the *Alphaherpesvirinae* subfamily.

Despite the presence of anti-LETV antibodies in wild turtles, clinical signs of LETD (Coberley et al. 2001b) and any DNA based evidence of LETV have not been detected in free-ranging turtles in Florida. The present study sought to detect LETV DNA in the offspring of nesting adult females that are shown to have been exposed to the virus, and therefore could potentially shed the virus.

This study tested the hypothesis that LETV is vertically transmitted in nesting green and loggerhead turtles (All turtles originating from the ACNWR, Florida). Plasma samples from nesting turtles were tested for anti-LETV antibodies using ELISA. Oviductal fluids and eggs from turtles with high anti-LETV levels were tested for the presence of the LETV DNA POL using PCR primers for POL. Degenerate PCR primers that amplify most herpesviral DNA POL genes were also used in this study. Results show high anti-LETV antibody reactivity in both loggerhead and green turtles. PCR results indicate absence of LETV and other herpesviral DNA in the oviductal fluid and eggs of LETV seropositive adults.

Methods

Sample Collection

Samples (plasma, oviductal fluid, and eggs) were collected from 15 nesting loggerhead turtles from May-August 2001, and two FP positive green turtles (plasma,

eggs) from May-August 2000 at the ACNWR, Florida. Each nesting turtle encountered was tagged, and morphometric data were collected. The location of each nest was marked. During oviposition, oviductal fluid of loggerhead turtles was collected in sterile 50 ml tubes and promptly frozen. Post-oviposition, blood was collected from all nesting turtles using a 1.5 inch 21 gauge needle inserted into the dorsal cervical sinus (Owens and Ruiz 1980). Blood was centrifuged to separate red blood cells from plasma. Plasma was separated into cryovials and frozen. Seventy days post oviposition, unhatched eggs and dead hatchlings were collected from each nest using sterile plastic bags labeled with the following information: turtle tag numbers, date nest was laid, and date of inventory. To prevent cross-contamination, new latex gloves were used to inventory each nest. All eggs present were collected, both hatched and unhatched, and were placed into a sieve. Hatched eggs were counted, and then placed back in the nest, while unhatched eggs and dead hatchlings were counted, deposited into a prelabeled bag and placed in a freezer.

Antibody assay

All plasma samples (n=17) were sent to the laboratory of Dr. Paul Klein at the University of Florida for determination of the presence of anti-LETV antibodies using ELISA. All plasma samples were evaluated at 1:25 dilution (Coberley et al. 2001a).

DNA isolation

DNA was isolated from oviductal fluids and eggs of turtles with high anti-LETV antibodies. For loggerhead turtle samples, 9 oviductal fluid samples (one sample from

each turtle), and 16 egg samples (2 eggs from each turtle) were dissected using sterile instruments for DNA isolation (n=25 total DNA samples). For the green turtle samples, 4 eggs and one fetus were dissected for DNA isolation (n=8 total DNA samples acquired). Including both species, DNA was isolated from a total of 33 samples for this study. All samples were digested in 500 µl Rosetti's Buffer (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 0.6% SDS, 25 mM EDTA pH 8.0), .2 mg Proteinase K, and 10 mg trypsin for 48 hours at 37° C. After digestion, each sample was extracted 2 times with 500 µl phenol, vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and was extracted 2 times with 500 µl of PCI (50 phenol:48 chloroform:2 isoamyl alcohol), vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and was extracted 2 times with 500 µl CI (48 chloroform:2 isoamyl alcohol), vortexed, and centrifuged at 13,000 X G for 5 minutes. Supernatant was transferred to a new tube, and DNA was precipitated with 1/10 final volume 3M sodium acetate and 2 X final volume 100% ice cold EtOH. Samples were placed on ice for 60-120 minutes, washed with 70% ice cold EtOH, air dried, and resuspended in 200 µl 1X TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0). DNA samples were quantified on a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) and visualized on a 1.1% agarose gel (Fig 6).

Amplification and sequencing

An array of strategies were employed to screen turtle DNA samples, via PCR amplification, of the DNA POL gene of LETV. Standard primer pair amplification

(LETV1/LETV2) was used in addition to nested PCR using degenerate primers which amplify most herpesviruses (Table 3). Variations in PCR reaction chemistry were tested to identify optimal reaction ingredients for nested and standard PCR. A diversity of thermocycling parameters was also tested to optimize amplification conditions (Table 4, Appendix). All products resulting from PCR amplification were sequenced and compared to known sequences using NCBI BLAST algorithm.

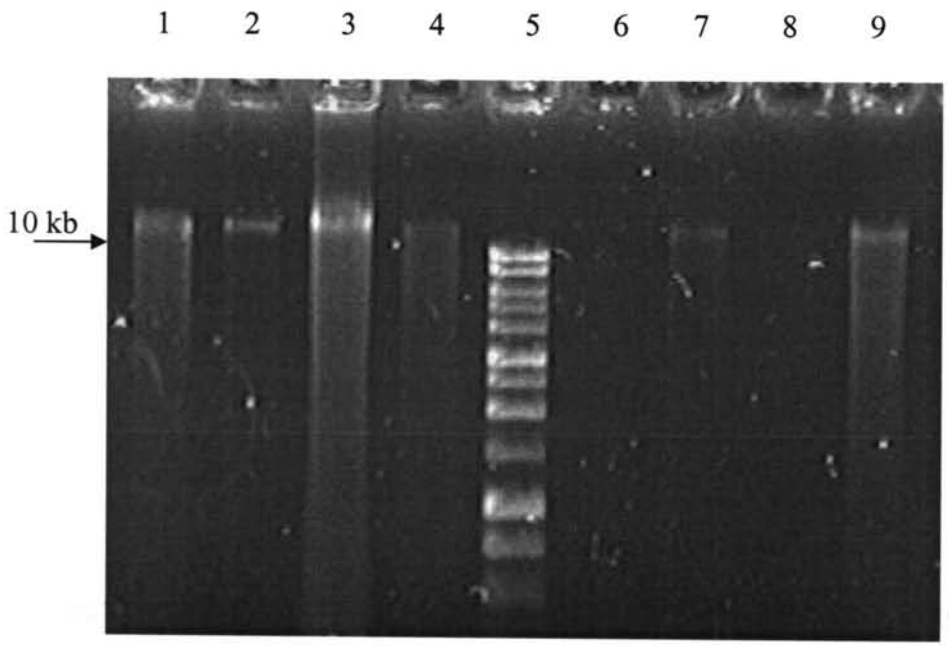

Figure 6. Products of DNA isolation from oviductal fluid and egg membranes. Lane 1: (N9121) oviductal fluid; Lane 2: (W0136) oviductal fluid; Lane 3: (W0828) oviductal fluid; Lane 4: (W0859) oviductal fluid; Lane 5: Promega 1 kb ladder; Lane 6: (W0805) inner membrane of egg; Lane 7: (W0668) inner membrane of egg; Lane 8: (W0859) inner membrane of egg; Lane 9: (W0670) inner membrane of egg.

Table 3. PCR primer sequences (5'-3'); Ambiguous codes follow IUB standards

Primer	Sequence
PAK1	GTCCTTGCTACCANTCNACNCCYTT
PAK2	TGTAACTCGGTGTAYGGNTTYACNGGNGT
PAK3	CACAGAGTCCGTRTCNCCRTADAT
PAK6	TCCTGGACAAGCRNYSGCNMTNAA
LETV1	TGCAATTCGGTGTACGGCTTC
LETV2	CACGGAATCCGTGTCGCCGTA
CR1	TTGTACATCTACTTATTTACCAC
CR2	GTACGTACAAGTAAAACTACCGTATGCC

Table 4. PCR protocols used for each set of primers (See Appendix for details)

PCR primers	Protocol # used
PAK1/PAK6/PAK2/PAK3	1, 6, 7
LETV1/LETV2	2, 3, 4, 8, 9
CR1/CR2	10

A range of DNA (1-800ng), dNTP (.3-.8mM), and magnesium chloride (1.5-4mM) concentrations, plus the inclusion and exclusion of 1% DMSO, were used in combination with the different cycling conditions (Appendix). Also, different brands of *Taq* polymerase were tested (Sigma, St. Louis, MO; and Bioline, Randolph, MA), each with different buffers (KCl and NH_4 respectively). A positive and a negative control (deionized water) were used in each round of PCR.

An initial set of nested PCR reactions using two sets of degenerate primers (PAK1/PAK6 and PAK2/PAK3) at a final concentration of .5 μM per primer was conducted (Fig 7). The primers (PAK2/PAK3) used in the second round of PCR amplify a 224 bp conserved region of the herpesviral DNA POL gene (VanDevanter et al. 1996). Varicella zoster virus (VZV) DNA (100 ng) was used as a positive control in these reactions. PCR was performed on all samples using specific primers (LETV1/LETV2; .1 μM each primer), designed for a 224 bp region of the LETV DNA POL gene (Fig 7). Approximately 20 ng of DNA from an infected turtle cell lysate was used as a positive control in these reactions. Sizes of PCR products were determined on a 1.5% agarose gel with either AmpliSize (Bio-Rad, Hercules, CA) or pGEM (Promega, Madison, WI) DNA ladder and visualized using ethidium bromide staining.

As an added positive control to assess the quality of isolated DNA, 4 DNA samples were used in PCR reactions using the primer pair CR1/CR2, which amplify a 400 bp region of the mitochondrial d-loop region of green and loggerhead turtles (Norman et al. 1994). In each 25 μl reaction, .04 mM dNTPs, .5 μM each primer,

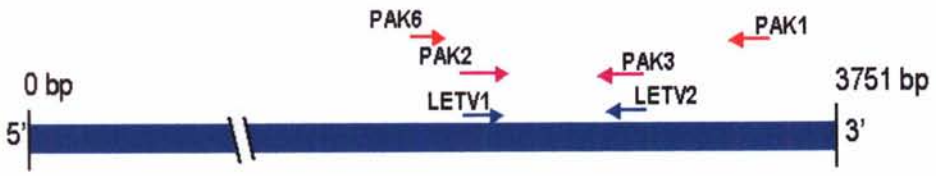

Figure 7. PCR primers used for the amplification of the herpesviral DNA POL gene

1.5 mM magnesium chloride, .1 µl DMSO, 2.5 µl Sigma buffer (St. Louis, MO) and .5 units Sigma *Taq* (St. Louis, MO) were used.

Positive PCR products were gel purified and isolated using the Qiagen MinElute Gel Extraction Kit (Qiagen Inc., Valencia, CA). Purified PCR products from the mtDNA assays were quantified on a 1.2% agarose gel using Low Mass Ladder (Invitrogen, Carlsbad, CA). These products were directly sequenced using 2 µM primers, 20 ng PCR product, and 2 µl Quick Start Ready Reaction Mix from the CEQ Dye-Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA). Cycling conditions were 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 minutes, for a total of 34 cycles. Sequencing reactions were centrifuged at 4000 rpm for 20 seconds, and 2.5 µl of stop solution (1µl 3M sodium acetate pH 5.2, 1 µl 100 mM EDTA pH 8.0, 0.5 µl 20 mg/ml glycogen) was added to each reaction. Sequencing reactions were precipitated with 60 µl ice cold 95% EtOH, sealed, inverted vigorously 5-10 times, and placed at –20° C for 10 minutes. The samples were then centrifuged at 4000 rpm for 10 minutes. The plate was inverted to remove the supernatant and centrifuged upside down at 300 rpm for 20 seconds. The pellets were rinsed 2 times with 200 µl ice cold 70% EtOH. Following each rinse, the plate was centrifuged at 4000 rpm for 3 minutes, inverted to remove the supernatant, and centrifuged upside down at 300 rpm for 20 seconds. The samples were then vacuum dried for 10 minutes, resuspended in 20 µl SLS buffer (Beckman-Coulter, Fullerton, CA) and separated on a Beckman CEQ2000 automated sequencer according to manufacturer's protocols. Raw sequence chromatographs were edited using Sequecher

4.1 (Gene Codes, Ann Arbor, MI), and sequence identity was inferred by searching the NCBI databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Purified PCR products other than the mtDNA samples were cloned to search for the possibility of multiple herpesviruses in the PCR products. Cloning was completed by using Topo TA cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Multiple clones from a single PCR product were grown overnight in liquid culture (LB plus ampicillin). Plasmids with inserts were then purified using the QiaPREP Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Plasmids were sequenced using M13 primers with the CEQ Dye-Terminator Cycle Sequencing Quick Start Kit (Beckman-Coulter, Fullerton, CA). Each reaction contained approximately 300 ng of plasmid DNA, 4 pmol of each primer, and 2 μl of Quick Start Ready Reaction Mix (Beckman-Coulter, Fullerton, CA). Samples were sequenced, edited and identified as described above.

Results

Nine of 15 loggerhead plasma samples (60%), and 2 of 2 green turtle samples (100%) were positive for presence of high antibody levels to LETV (Table 5). Optical density (OD) readings greater than .310 were considered positive. This is an arbitrary cut-off value previously calculated based on optical density readings of 42 healthy captive raised green turtles (Coberley et al. 2001a).

Table 5

ELISA readings for detection of anti-LETV antibodies in nesting green (Cm) and loggerhead (Cc) turtles

Turtle ID	Species	OD Reading	LETV positive/negative
N9121	Cc	0.404	positive
W0136	Cc	1.349	positive
W1038	Cc	0.131	negative
W0668	Cc	1.726	positive
W0670	Cc	2.413	positive
W0805	Cc	2.023	positive
W0809	Cc	0.219	negative
W0815	Cc	0.097	negative
W0828	Cc	2.182	positive
W0831	Cc	2.703	positive
W0859	Cc	1.946	positive
W0861	Cc	0.095	negative
W1034	Cc	2.444	positive
W1073	Cc	0.072	negative
W0811	Cc	0.074	negative
X8874	Cm	0.525	positive
X8907	Cm	0.352	positive

Eight of 9 oviductal fluid samples, 4 of 16 loggerhead egg samples, and 1 of 8 green turtle egg samples produced light smears at approximately 224 bp using nested PCR under protocol 1 (Appendix). BLAST search indicated these sequences to be insignificant, as none of them showed similarity to any herpesvirus. The sequences were multiple tandem primer sequences. Positive and negative controls worked in all PCR assays.

All 33 DNA samples (eggs and oviductal fluid) produced negative results using LETV PCR primers (Fig 8 and Fig 9). Positive and negative controls worked in all PCR assays.

Three of 4 DNA samples (2 different oviductal fluid samples, and one internal membrane of an egg) produced positive results using d-loop mtDNA primers under protocol 10 (Appendix; Fig. 10). BLAST search indicated these sequences were homologous to the loggerhead (*Caretta caretta*) mitochondrial 5' control region (Score 662, E-value 0).

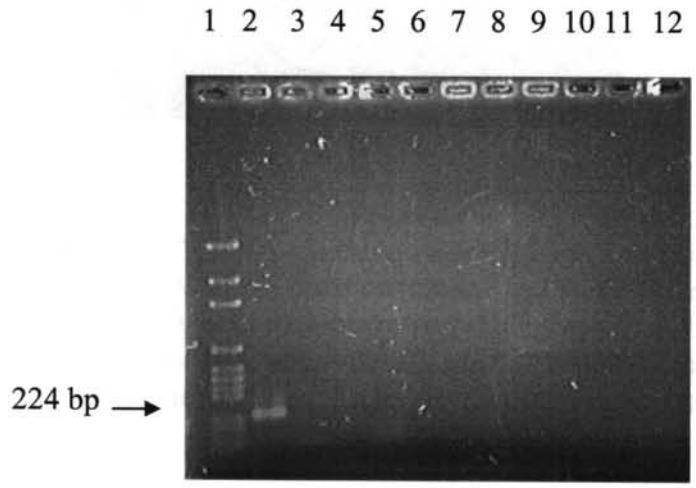

Figure 8. Absence of LETV DNA POL in oviductal fluid using LETV specific primers; Lane 1: pGEM marker; Lane 2: LETV DNA (positive control); Lane 3: deionized water (negative control); Lane 4: N9121; Lane 5: W0136; Lane 6: W0668; Lane 7: W0670; Lane 8: W0805; Lane 9: W0828; Lane 10: W0831; Lane 11: W0859; Lane 12: W0134

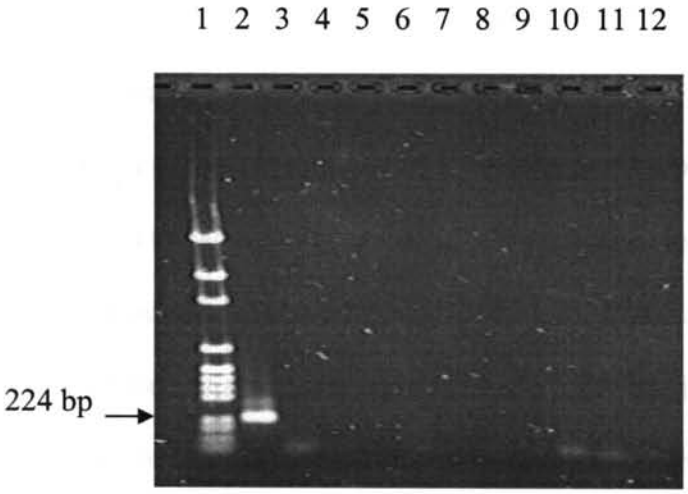

Figure 9. Absence of LETV DNA POL in eggs using LETV specific primers; Lane 1: pGEM marker; Lane 2: LETV DNA (positive control); Lane 3: deionized water (negative control); Lane 4: (W0828) inner membrane and shell; Lane 5: (W0828) inner membrane; Lane 6: (W0831) inner membrane and shell; Lane 7: (W0831) inner membrane; Lane 8: (W0805) inner membrane; Lane 9: (W0805) inner membrane; Lane 10: (W0668) inner membrane and shell; Lane 11: (W0668) inner membrane; Lane 12: (W0859) inner membrane

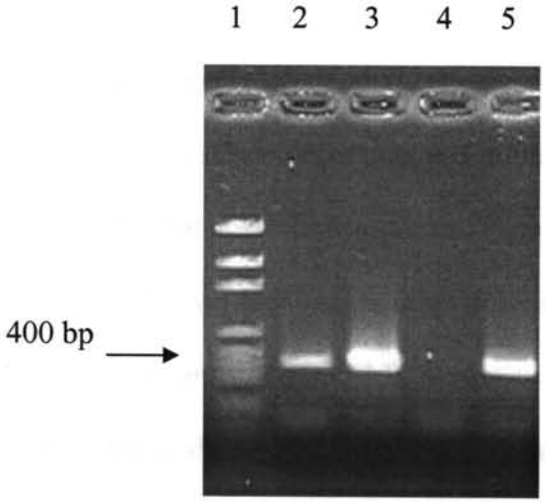

Figure 10. Amplification of *Caretta caretta* d-loop mitochondrial gene in oviductal fluid and eggs. Lane 1: pGEM size marker; Lane 2: (W0668) oviductal fluid; Lane 3: (W0136) oviductal fluid; Lane 4: (W0831) internal membrane of egg; Lane 5: (W0859) internal membrane of egg.

Discussion

Lung-eye-trachea disease was first noted in captive turtles in 1975 (Jacobson et al. 1986). To date, most of the knowledge of LETV infection stems from immunological work that determined the seroprevelance of LETV antibodies in wild green and loggerhead turtle populations (Coberley et al. 2001 a,b). Clinical signs of LETD were not observed in the present study, and have yet to be described in wild turtles in Florida.

The results from the ELISA in the present study indicate that wild marine turtle populations are being exposed to LETV, thereby increasing the seroprevelance of this disease in free-ranging marine turtles. LETD was first documented in mariculture turtles (Jacobson et al. 1986), so recent evidence of exposure to a LETD-associated herpesvirus in free-ranging turtles brings up concerns over the possibility of the release of diseased turtles from captivity. The serological based evidence also provides greater insight into the diversity of herpesviruses that affect marine turtles on Florida's east-central coast.

In the present study, LETV DNA was not detected in eggs and oviductal fluid of nesting turtles with high antibody levels, suggesting absence of vertical transmission of LETV, or if vertical transmission is occurring, it is quite rare. Also, it is likely that detection of the virus was not possible with the methods utilized in the present study. The absence of evidence for vertical transmission presented in this study indicates that the question of horizontal transmission of LETV in marine turtles warrants further investigation. For example, damselfish neurofibromatosis was transmitted in a density dependent fashion (Schmale 1991). Large congregations of marine turtles at nesting or foraging grounds may increase transmission of disease. Nesting turtles migrate from

their nesting grounds to feeding grounds, so it is uncertain where this virus is being transmitted. Florida turtles feed in bays and lagoons where run-off is substantial and there is low flushing (Herbst 1994). These habitats are susceptible to build-up of infectious agents, and if turtles are in contact with one-another at these sites, disease transmission is likely to occur. Curry et al. (2000) also showed that LETV can remain infectious in seawater for up to 5 days. The combination of a habitat susceptible to high concentrations of infectious viruses, and the stability of LETV can prove detrimental to populations of marine turtles.

While the results of this study failed to detect evidence of vertical transmission of LETV, it cannot be ruled out that this route of transmission may be occurring, albeit rarely. Only a few eggs from each nest were used for DNA isolation due to the number of unhatched eggs remaining at inventory (seventy days post-oviposition). The possibility also remains that viral DNA is present in eggs that were not tested or viral DNA is present in tissues of hatchlings, such as the gonads, that were not examined. The mtDNA PCR assay validated the quality of most samples used in the present study. However, the one sample that produced negative results in this assay may have been degraded. An agarose gel showing results of the DNA isolation of this sample indicated low quantity of DNA.

In the present study, I used antibody positive adult females because these were known to be LETV exposed, since this is currently the only marker for potential infection. Exposure, however, does not indicate whether or not the mother is still infected and still capable of shedding infectious virus. In fact, it is possible that females

with high antibody concentrations have cleared the infection or effectively inactivating any virus that is shed during oviposition if in fact the vertical transmission mechanism is due to shedding virus particles in the reproductive tract. However, if the mechanism of vertical transmission is due to genetic transfer via oocyte or sperm, then antibodies are irrelevant.

Future studies may examine the transmission of LETV in a larger sample set of eggs in each nest if possible, and consider a larger number of nesting turtles. Although PCR methodology is a powerful method of detection, alternate techniques, such as the use of Southern blots, could also be used to validate positive PCR products obtained from the offspring of diseased mothers. Further studies are critical to learning more about LETD and its affect on wild turtle populations. Antibody-based tests of nesting turtles could examine the hypothesis of maternal transfer of antibodies to offspring. Evidence of maternal antibody transfer has been noted in desert tortoises (*Gopherus agassizii*; Schumacher et al. 1999) and Blue and Gold Macaws (*Ara ararauna*; Lung et al. 1996).

This novel work provides information on the exposure to a relatively unstudied disease (LETD) in marine turtle populations, as well as characterizing the absence of vertical transmission of LETV. Further investigations on the modes of transmission, presence or absence of DNA based evidence, and observations of clinical signs of LETD in free-ranging marine turtles are needed to fully characterize the pathology of this disease. Examining the role of certain ecosystems in the prevalance of LETV may aid in further determining the route of transmission of this disease. Conservation strategies, such as vaccine development, can then be implemented to lessen the spread of LETV,

and possibly other disease-associated herpesviruses afflicting marine turtles. This study contributes significantly to the current knowledge of LETD, and may aid in the long-term management and survival of endangered and threatened marine turtles worldwide.

CHAPTER 4: DISCUSSION

Disease is one of many factors that currently impacts populations of marine turtles worldwide. The causative agents of diseases are many, however, the focus of this research was on the spread of herpesviral diseases in marine turtles. Herpesviruses have been associated with diseases in a suite of vertebrates (Fields et al. 1996), including several species of marine turtles. Horizontal transmission of FPHV has been shown to occur in marine turtles (Herbst et al. 1995, Herbst et al. 1996). The role of vertical transmission (the spread of a pathogen from one generation to another) of herpesviruses has also been investigated in amphibians, birds, fish, humans, and reptiles, but has yet to be examined in marine turtles. The present studies attempted to find evidence of vertical transmission in the spread of two separate disease-associated herpesviruses in marine turtles, FPHV and LETV.

The first study examined the role of vertical transmission in the spread of FPHV in nesting green turtles afflicted with FP at the ACNWR, Florida. FPHV is detected in turtles afflicted with FP; therefore, if this virus is vertically transferred, it is more likely to be detected in tissues of the offspring of FP positive females. Results of the present study did not support vertical transmission of FPHV, suggesting that this disease may be transmitted in an alternate fashion (e.g. horizontally), and has the potential to still be vertically transmitted as well, albeit rarely. Control experiments including the successful isolation, amplification, and sequencing of FPHV DNA POL from a FP tumor to validate the methods used in these experiments, and the amplification and sequencing of a

mitochondrial marine turtle gene in DNA samples originating from offspring indicate the high quality of DNA used in the study.

The second study investigated the role of vertical transmission in the spread of the LETV in nesting green and loggerhead turtles at the ACNWR, Florida. There is currently no DNA based evidence for LETD in wild populations of marine turtles in Florida; the only evidence for LETD in wild populations is from immunological studies. An ELISA was developed by Coberley et al. (2001a) that detected antibodies to LETV in both free-ranging green (Coberley et al. 2001a) and loggerhead turtles (Coberley et al. 2001b). In the present study, DNA from offspring and oviductal fluid of turtles with high anti-LETV levels were tested for the presence of LETV. Results failed to demonstrate evidence of vertical transmission of LETV in both the oviductal fluid and offspring. In a control experiment, I amplified a marine turtle mitochondrial gene in the DNA samples used in this study, which validates the quality of this DNA.

Although the present studies provided no evidence of vertical transmission of marine turtle herpesviruses, future work on transmission of these diseases is necessary. Although PCR is sensitive, alternative techniques, such as Southern blot, could be employed to validate positive PCR products obtained from the offspring of diseased turtles. Future studies examining greater numbers of nests, eggs, and/or tissue types may provide additional evidence favoring the rejection of the hypothesis of vertical transmission of these herpesviruses. Future studies, which examine incidence of horizontal transmission of herpesviruses, may be the most critical to our eventual understanding of the epizootiology of both LETD and FP. Characterization of the

environments in which diseased animals occur may also aid in the etiology of these diseases. These studies and others at the molecular and immunological levels will identify modes and patterns of pathogenesis, and allow for the meaningful and scientifically grounded conservation measures to be taken to address the spread of marine turtle diseases worldwide. Determining the commonality of vertical transmission versus horizontal transmission will affect the use of vaccines as possible modes of disease control, and may ultimately aid in the survival of these endangered species.

APPENDIX

PCR Protocols

1. Degenerate PCR:
    1. 94° 0 min
    2. 94° 1 min
    3. 46° 1 min
    4. 72° 1 min

    Steps 2-4 repeated 44 more times

    5. 72° 10 min.

2. Specific PCR:
    1. 94° 5 min
    2. 94° 30 sec.
    3. 62° 30 sec
    4. 72° 30 sec.

    Steps 2-4 repeated 34 more times

    5. 72° 5 min.

3. Specific Gradient PCR #1:
    1. 94° 5 min.
    2. 94° 30 sec.
    3. 55° to 70° for 30 sec.
    4. 72° for 30 sec.

    Steps 2-4 repeated 34 more times

    5. 72° 5 min

4. Specific Gradient PCR #2:
    1. 94° 5 min.
    2. 94° 30 sec.
    3. 50° to 65° for 30 sec.
    4. 72° 30 sec.

    Steps 2-4 repeated 34 more times

5. Degenerate Step PCR #1:
    1. 95° 3 min.
    2. 94° 1 min.
    3. 44° 30 sec.
    4. 68° 45 sec.
    5. 94° 1 min.
    6. 54° 30 sec.

        -.3° per cycle

    7. 72° 45 sec.

    Steps 5-7 repeated 44 more times

    8. 72° 10 min.

6. Degenerate Step PCR #2:
    1. 95° 3 min.
    2. 94° 1 min.
    3. 44° 30 sec.
    4. 68° 45 sec.
    5. 94° 1 min.
    6. 54° 30 sec.
        -.2° per cycle
    7. 72° 45 sec.

    Steps 5-7 repeated 44 more times

    8. 72° 10 min.

7. Degenerate Step PCR #3:
    1. 95° 3 min.
    2. 94° 1 min.
    3. 44° 30 sec.
    4. 68° 45 sec.
    5. 94° 1 min.
    6. 52° 30 sec.
        -.1° per cycle
    7. 72° 45 sec.

    Steps 5-7 repeated 44 more times

    8. 72° 10 min.

8. Specific Step PCR #1:
    1. 95° 3 min.
    2. 94° 1 min.
    3. 65° 30 sec.
    4. 68° 45 sec.
    5. 94° 1 min.
    6. 65° 30 sec.
        -.2° per cycle
    7. 72° 45 sec

    Steps 5-7 repeated 44 more times

    8. 72° 10 min.

9. Specific Step PCR #2:
    1. 95° 3 min.
    2. 94° 1 min.
    3. 65° 30 sec.
    4. 68° 45 sec.
    5. 94° 1 min.
    6. 68° 30 sec.
        -.2° per cycle
    7. 72° 45 sec.

    Steps 5-7 repeated 44 more times

    8. 72° 10 min.

10. D-loop mtDNA PCR:
    1. 93° 2 min. 50 sec.
    2. 92° 30 sec.
    3. 52° 30 sec.
    4. 72° 30 sec.

    Steps 2-4 repeated 39 more times

    5. 72° 10 min.

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