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DISTRIBUTION OF CHELONID FIBROPAPILLOMATOSIS-ASSOCIATED HERPESVIRUS VARIANTS IN FLORIDA: MOLECULAR GENETIC EVIDENCE FOR INFECTION OF TURTLES FOLLOWING RECRUITMENT TO NERITIC DEVELOPMENTAL HABITATS

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DISTRIBUTION OF CHELONID FIBROPAPILLOMATOSIS-ASSOCIATED HERPESVIRUS VARIANTS IN FLORIDA: MOLECULAR GENETIC EVIDENCE FOR INFECTION OF TURTLES FOLLOWING RECRUITMENT TO NERITIC DEVELOPMENTAL HABITATS

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ABSTRACT: Marine turtle fibropapillomatosis is associated with chelonid fibropapilloma-associated herpesvirus (C-FP-HV) and commonly affects juvenile green turtles (*Chelonia mydas*) in neritic (nearshore) habitats. Green turtles have a complex life history, characterized by shifts in trophic level as well as habitat during ontogeny. Thus, several hypotheses can be proposed for when turtles become infected with C-FP-HV. They may acquire the virus at an early stage in the life cycle, including prenatal, hatchling, or the posthatchling pelagic stages. Alternatively, they may become infected later in life after they emigrate from the open ocean to neritic habitats. Each hypothesis generates predictions about the spatial distribution of genetic variants of C-FP-HV among nearshore sites within a region. Sequencing of polymerase chain reaction–amplified viral DNA from fibropapillomas of individual turtles was used to genotype the viral variants present in marine turtles from different coastal areas in Florida. We found four distinct virus variants (A, B, C, and D), two of which (A and C) were present in multiple turtle species. Green turtles in Florida were infected with variants A, B, and C. Variant A was found in green turtles from all three areas. Outside the Indian River Lagoon, variant A was most commonly detected and was found in >94% of diseased green turtles and 70% of loggerhead sea turtles (*Caretta caretta*) in the Florida Bay/Florida Keys. However, in the Indian River Lagoon, variant B was found in >94% of affected green turtles. Variant B was not detected outside of the Indian River system. Chi-square analysis strongly supported ($P<0.001$) an association between viral variant distribution in green turtles and location. On the basis of the assumption that juvenile green turtles found in Florida's west-central coast, Florida Keys, and Indian River Lagoon areas represented recruits from a mixed pelagic population, we expected that the distribution of viral variants in these turtles would be relatively homogeneous among locations; this would correspond to infection in the earlier phases of their life cycle. The heterogeneous distribution of viral variants in green turtle tumors from different Florida coastal locations strongly supports the hypothesis that, during epizootics, turtles are infected with specific C-FP-HV variants after they arrive as juveniles in neritic habitats. The conclusion that C-FP-HV is acquired after turtles recruit to nearshore habitats should help focus further research efforts on understanding the mechanisms of transmission and raises the possibility that the effect of fibropapillomatosis on turtle populations might be reduced by management strategies designed to break the cycle of transmission in these locations.

Key words: C-FP-HV, Chelonid fibropapilloma-associated herpesvirus, epidemiology, fibropapillomatosis, Florida, green turtle, herpesvirus, loggerhead turtle, marine turtle.

INTRODUCTION

Fibropapillomatosis (FP) is a debilitating neoplastic disease that is known to cause morbidity and mortality in marine turtles in various locations around the world. Affected turtles can develop multiple tumors ranging in size up to 20 cm in diameter. Tumors located on the flippers, axillary or inguinal regions, neck, mouth, or head can disrupt locomotion, feeding or vision. In some cases, the tumors occur as visceral fibromas that can lead to organ failure and death. In the past two decades, FP has emerged as a panzootic disease in green turtles (*Chelonia mydas*) and other endangered marine turtle species (Balazs and Pooley, 1991; Herbst et al., 2004; Jones, 2004). It is a major factor in stranding and mortality

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among green turtles, particularly in coastal waters around Florida (Foley et al., 2005). When FP was first described in 1938, the tumor prevalence observed in green turtles collected in the Key West fishery of Florida was 1.5% (3 of 200 animals) (Smith and Coates, 1938). More recently, disease incidence increased dramatically and has remained high over the past two decades (Herbst, 1994). In some localities, such as the Indian River Lagoon, Florida Bay, and the Florida Keys, from 50% to 70% of the green turtles are affected. Fibropapillomatosis also affects loggerhead sea turtles (*Caretta caretta*) and Kemp's ridleys (*Lepidochelys kempii*) in Florida waters, although it is less common in these species. Understanding the epidemiology of FP is an important component for the successful management of marine turtle populations.

Fibropapillomatosis is associated with a novel alphaherpesvirus called chelonid FP-associated herpesvirus (C-FP-HV) (Herbst et al., 2004), which is present in 100% of naturally occurring tumors. The virus is also present in 100% of tumors induced by inoculation of captive-reared green turtles with cell-free filtrates of natural tumors (Herbst et al., 1995; Herbst et al., 1996). We recently reported the use of inverse polymerase chain reaction (PCR) to characterize more than 43,000 bp of the C-FP-HV genome (Herbst et al., 2004). By sequencing segments of the viral genome from multiple individual, free-ranging turtles, we found variants of C-FP-HV exist that have up to 5.6% nucleotide sequence diversity. On the basis of predicted mutation rates, we concluded that at least four lineages leading to contemporary C-FP-HV variants evolutionarily diverged millions of years ago. Therefore, the emergence of fibropapillomatosis epizootics at multiple locations around the world during the past two decades is unlikely to be due to recent virulence mutations in the virus because it is highly improbable that such mutations could occur independently in four lineages. It is far more likely that changes in the environment or ecological factors that affect virus transmission or disease expression explain the recent upsurge in disease prevalence.

This raises the question of which environmental factors are more important in determining FP prevalence. Are disease outbreaks primarily determined by the presence and transmission of the virus? Alternatively, is virus infection less limiting and FP prevalence primarily determined by cofactors affecting host susceptibility and disease expression? To begin to address this issue, it is critical to determine at what point within a turtle's life cycle it may become infected and in what geographical locations infection occurs. Evidence indicates that after dispersing from their natal beaches, posthatchling sea turtles spend several years in the pelagic (open ocean) environment before recruiting to neritic (nearshore) developmental feeding habitats (Bolten, 2003). Fibropapillomatosis is detected primarily in juvenile and immature sea turtles in these coastal habitats (Herbst et al., 2004). It is unclear whether posthatchling pelagic phase turtles in the wild are infected and ever develop FP before recruitment to neritic habitats. In laboratory-infected animals, clinically apparent FP develops 2 to 6 mo after intradermal injection with C-FP-HV (Herbst et al., 1995; Herbst et al., 1996). Thus, the appearance of tumors in juvenile turtles after recruitment to neritic habitats could be due to either a prolonged tumor developmental period following infection or to environmental conditions encountered in nearshore waters that trigger or promote tumorigenesis. The distribution of genetic variants of C-FP-HV among localities and habitats in a region may be informative for elucidating the location and life history stage that turtles become infected.

Several possibilities exist for when natural virus infection of free-ranging turtles occurs, and each leads to a different prediction about the distribution of virus variants found in nearshore, developmental

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habitats. Infection with C-FP-HV could occur either in the natal beach environment (maternal shedding), posthatchling pelagic environment, or after recruitment of juveniles to neritic environments. If turtles are predominantly infected on the natal beach, then they could carry the latent infection to the pelagic environment where they potentially mix with posthatchlings from other nesting beaches. If the mixed population of C-FP-HV infected pelagic turtles recruits to neritic habitats in a nonassortative manner, then the frequency distribution of virus variants found in turtles in these neritic habitats should reflect the distribution of variants in the pelagic population. One would not expect significant heterogeneity in the frequencies of virus variants among localities on a regional scale. However, if juvenile turtles exhibit some degree of natal philopatry, selecting feeding areas that are close to their natal beach, then regional heterogeneity in virus variants found in these localities could develop. If turtles are primarily exposed to C-FP-HVs in the pelagic environment, one expects the virus variants found in recruits to nearshore habitats to be a representative sample of those encountered in the pelagic environment and relatively homogenous with respect to nearshore locality, regardless of whether or not juveniles show assortative (philopatric) or essentially random recruitment to these localities. Finally, if infection occurs after juveniles recruit to specific nearshore localities, the virus variants detected are those that are present in that habitat. The relative frequencies of these virus types could vary extensively from site to site, depending on turtle movements and hydrologic conditions. It would even be possible for unique endemic variants to emerge depending on epizootic genetic founder effects.

To distinguish among these possibilities, we examined the viral variants present in samples from two sympatric sea turtle species, green turtles and loggerheads, to determine the relative frequencies of each variant in three different coastal areas in Florida.

MATERIALS AND METHODS

Samples

Turtles affected with fibropapillomatosis were either live-captured during population studies and released or presented as live strandings to the Turtle Hospital, Marathon, Florida, USA, for rehabilitation. Tumor samples were collected immediately following capture or within a few days of recovery. Tissues were collected using sterile surgical instruments and immediately frozen at ≤ -20 C. When individual turtles presented with multiple tumors, several tumor biopsies were pooled for analysis. Nucleic acids were extracted following proteinase K digestion and genomic DNA isolation (Qiagen Genomic-tip; Qiagen, Valencia, California, USA) following the protocol of the manufacturer. Tumor samples were collected between 1990 and 2004.

Genetic analysis

To determine the distribution of the C-FP-HV variants in fibropapilloma-bearing turtles within Florida waters, viral sequences were PCR-amplified from tumor DNA (Fig. 1). A previous phylogenetic study (Herbst et al., 2004) compared a total of 6,801 bp sequenced from five PCR-amplified segments (called amplicons I through V) of the unique long region of the C-FP-HV genome among different affected turtles. This analysis identified a novel 4 kb region between the UL18 and UL15B genes that was highly polymorphic among C-FP-HV variants. There is no homolog of the 4 kb region in other alphaherpesviruses. Two PCR products within this region, called amplicons IV and V (Herbst et al., 2004), were found to discriminate all the known C-FP-HV virus variants identified in that study and encompass all the differences between Florida variants A, B, and C. In this study, these two products were generated from each animal and sequenced. We amplified and sequenced a 1,213-bp region in amplicon IV, encompassing the carboxyl terminal portion of UL18, and a 1,353-bp segment of amplicon V. Primers were IV-F, 5′-AG GCCTGTATCTCCTGCTCA-3′ and IV-R 5′-TATCGCGAGCTCGTACAATG-3′ for amplicon IV and primers V-F, 5′-ACGGAGCGCAA TGTAGAGTT-3′ and V-R, 5′-TGAGTACGAG CCCGACTTCT-3′ for amplicon V. The amplicon V primers amplified the specific product from all samples tested (i.e., for all viral variants). For samples containing variant D, however, amplicon IV amplification required a dif-

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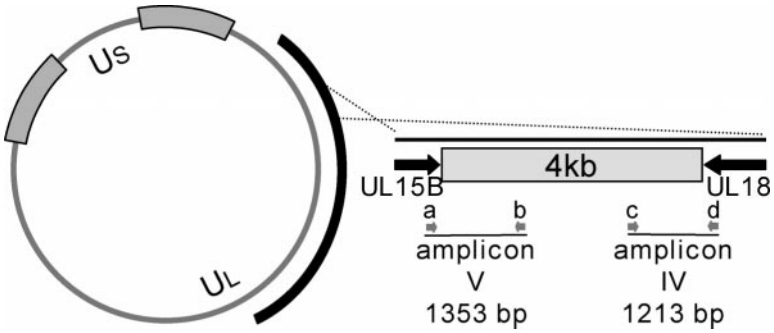


Figure 1. Chelonid fibropapilloma-associated herpesvirus (C-FP-HV) sequences analyzed. The circle shows that structure of alphaherpesvirus genomes including the unique short (US), unique long (UL), and repeated (gray boxes) segments. The outer black arc shows the 43,843-bp segment of the C-FP-HV for which the sequence has been published (GenBank accession number AY644454) (Herbst et al., 2004). A portion of the viral genome extending from the UL15B gene to the UL18 gene is shown expanded on the right. The 4-kb region that is not present in other alphaherpesviruses is shown. The positions of the PCR primers that were used to generate amplicons IV and V are shown. Numbers refer to the size of the sequenced segment for each amplicon.

ferent reverse primer, IV-R2 5′-TACCGTGA GCTCATGCAATG-3′. Polymerase chain was performed with Expand polymerase (Roche, Nutley, New Jersey, USA). Reaction conditions were 94 C for 2 min, followed by 10 cycles of 94 C for 10 sec, 58 C for 30 sec, and 68 C for 9 min, followed by 25 cycles under the same conditions, except that the 68 C elongation step was increased by 20 sec at each cycle. DNA products were purified and sequenced directly.

Sequences were aligned using the Clustal W algorithm in the MegAlign program (DNASTAR) and polymorphisms were identified. To confirm the turtle species, the sequence of the mitochondrial D-loop was also determined for each DNA sample using primers TCR5 and TCR6 (Norman et al., 1994). Distributions of relative frequencies of the four Florida variants were tested for heterogeneity among turtle species and among localities using chi-square statistics (Zar, 1974).

RESULTS

The present study analyzed tumors from 38 immature green turtles (ranging in size from 29 cm to 61 cm straight carapace length), 10 loggerheads (from 65 cm to 87 cm straight carapace length), and one Kemp's ridley turtle. Table 1 summarizes the numbers of turtles captured for each Florida coastal area and county represented in this study and the virus variants identified in their tumor samples. The species identity of every DNA sample used in the study was confirmed by PCR amplifying and sequencing, approximately 380 bp of the D-loop sequence of mitochondrial DNA (Norman et al., 1994).

Every viral sequence was precisely iden-

TABLE 1. Distribution of chelonid fibropapillomatosis (FP)-associated herpesvirus (C-FP-HV) variants among marine turtles with FP from three coastal areas in Florida.

Area	County	Species (*n*)	Virus variant			
			A	B	C	D
Florida west central coast	Citrus	Green turtle (2)	2	—	—	—
		Kemp's ridley (1)	—	—	1	—
	Sarasota	Green turtle (1)	1	—	—	—
Florida Keys / Florida Bay	Monroe	Green turtle (16)	15	—	1	—
		Loggerhead (10)	7	—	2	1
	Dade	Green turtle (2)	2	—	—	—
Indian River Lagoon	St. Lucie	Green turtle (6)	—	6	—	—
	Indian River	Green turtle (11)	1	10	—	—

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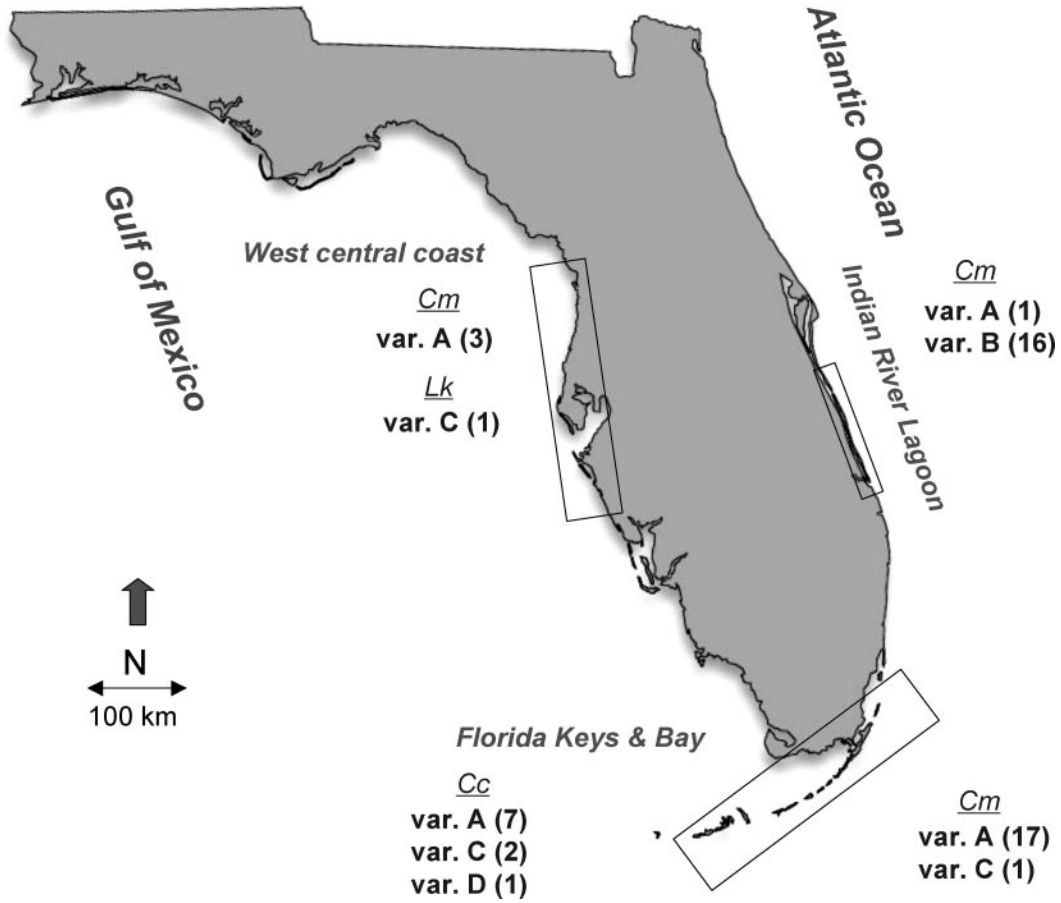


FIGURE 2. Distribution of C-FP-HV variants A, B, C, and D in coastal areas around Florida. Viruses were sequenced from turtle fibropapillomas acquired at three different locations indicated on the map. The distribution of the viral variants within the indicated species is shown for each area (*Cm=Chelonia mydas*—green turtle; *Cc=Caretta caretta*—loggerhead; *Lk=Lepidochelys kempii*—Kemp's ridley). Numbers show how many individuals were infected with the indicated strain.

tical to one of the previously described variants, A, B, C, or D (GenBank accession numbers AY646909 to AY646915 for amplicon IV and AY646916 to AY646922 for amplicon V). No additional mutations were detected. The most commonly detected variant was A. It was found in 28 of 49 total animals and was present in both green turtles and loggerheads. Variant C was detected in all three species examined, including two loggerheads, a green turtle, and the only Kemp's ridley sample. Variant D was detected in only a single loggerhead from Florida, although it was also previously observed in a loggerhead from North Carolina (Herbst et al., 2004). Within the Florida Bay/Florida Keys region, where both green turtles and loggerheads were represented, chi-square analysis did not demonstrate an association between viral variant and turtle species ($P=0.17$).

The distribution of the four viral variants in three different areas around Florida is shown in Figure 2. Variant A was found in all three localities represented in this sample. Outside of the Indian River Lagoon, variant A was the most prevalent variant detected, found in >94% of the green turtles and in 70% of the loggerheads in the Florida Bay/Florida Keys area. However, variant B was the predominant form in the Indian River Lagoon,

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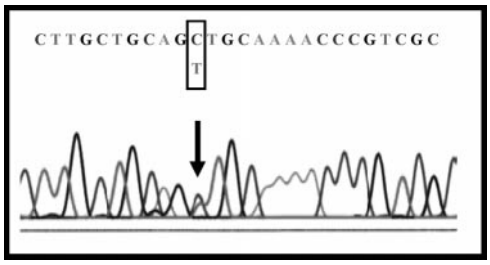


FIGURE 3. DNA sequencing chromatogram for amplicon IV of one green turtle from the Indian River. The arrow shows the position of the mixture of the nucleotides characteristic of variants A and B.

and it was detected exclusively in that location. Sixteen of 17 green turtles from the Indian River Lagoon were infected with variant B. One additional green turtle from this location was infected with variant A. Among green turtles from three locations, Florida west-central coast, Florida Bay/Keys, and Indian River Lagoon, there was a strong association between location and virus variant (chi-square, $P<0.001$). There was no association of green turtle mitochondrial DNA haplotype with either virus variant or location.

Interestingly, one of the Indian River green turtles was coinfected with variants A and B. Analysis of the DNA sequencing chromatogram of the amplicon IV product from this turtle indicated a mixture of nucleotides at the critical position that distinguishes variants A and B (Fig. 3). To confirm this, amplicon IV PCR products from this turtle were cloned in a plasmid vector. Ten individual clones were sequenced and both variants were detected. This is the first report that a wild turtle can be infected with more than one C-FP-HV variant.

DISCUSSION

Fibropapillomatosis is a significant threat to some marine turtles, and successful management of these species will require a solid understanding of the mechanisms of C-FP-HV transmission. This disease most commonly affects juvenile turtles in neritic developmental habitats (Herbst, 1994), but FP prevalence can vary substantially over short geographical distances (Herbst, 1994; Herbst and Klein, 1995). This prevalence variation could be explained if turtles are exposed to the etiological agent after arriving at these locations. A plausible alternative explanation for variation in disease prevalence is that the turtles are uniformly infected with the virus, but FP development is dependent on environmental cofactors that vary among local habitats (Herbst and Klein, 1995; Foley et al., 2005).

We analyzed the genetic variability of C-FP-HV in Florida coastal waters to test these alternative hypotheses about when in a turtle's life history virus transmission occurs. Variant A was ubiquitous and was the most prevalent C-FP-HV variant found in localities outside of the Indian River Lagoon. Variant B, however, predominated in the Indian River Lagoon and was not detected in any turtles captured elsewhere, suggesting that it is endemic to the lagoon and uncommon outside of that system. The significant heterogeneity in the distribution of virus variants among sites in Florida is not consistent with post-hatchling green turtles being exposed to C-FP-HV in the pelagic environment. These results are consistent with the hypothesis that infection occurs after juveniles recruit to specific nearshore localities, where the relative frequencies of viruses present can vary extensively from site to site, depending on turtle movements and hydrologic conditions.

Alternatively, these results are also consistent with the hypothesis that green turtles are infected at their natal beach, but only if latently infected pelagic turtles recruit selectively to neritic feeding areas that are close to their natal beach. If the relative frequencies of virus types vary significantly among natal beaches, the frequencies of virus variants present in the feeding area may also vary extensively from location to location. Natal philopatry is documented in nesting adult females of several species (Meylan et al., 1990; FitzSimmons et al., 1997). Adult male

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green turtles may also migrate to breeding grounds near their natal beach (Fitz-Simmons et al., 1997). There is some evidence that juvenile loggerheads recruiting from the pelagic environment to nearshore habitats may also exhibit some degree of natal philopatry (Bowen et al., 2004). However, the restriction of variant B to the Indian River Lagoon is difficult to reconcile with this hypothesis (natal beach transmission) because juvenile green turtles move extensively throughout coastal Florida (Foley et al., 2005). Several genetic studies of juvenile green turtles on feeding grounds in the Bahamas, Florida, and Barbados have shown that the populations are composed of mixed stocks (Lahanas et al., 1998; Bass and Witzell, 2000; Luke et al., 2004). Along the east coast of Florida, Bass and Witzell (2000) found nearly equivalent contributions from Costa Rica and Florida/Mexico rookeries in the population of foraging juvenile green turtles. It is difficult to imagine that juvenile turtles assort themselves among developmental habitats that they have never visited previously strictly as a function of natal beach origin, particularly over relatively short distances within a region such as the Atlantic Coast of Florida. Moreover, mitochondrial DNA sequences from our samples did not support any assortment of juvenile green turtles by matriline among localities in Florida.

Among the 6,801 bp of sequence from variant B, there is only a single base pair difference relative to variant A. This mutation is a silent, C to T transition on the coding strand of the UL18 gene near its carboxyl terminus. All the other viral variants, including isolates from Hawaii, match variant A at this position (Herbst et al., 2004). Variant A is the predominant form of C-FP-HV elsewhere in Florida waters. Thus, it is likely that variant B was formed by the C to T mutation on a variant A background. Consequently, we hypothesize that variant B is the result of a founder-effect mutation that occurred within the Indian River Lagoon and has been segregating by viral transmission among turtles within that system.

The geography of the Indian River Lagoon likely provided ideal conditions for finding heterogeneity in virus distribution. The Indian River Lagoon system is a series of three interconnected shallow estuarine lagoons extending approximately 250 km along the east coast of Florida. Water exchange with the Atlantic Ocean is restricted to only six inlets along the system's entire length. Thus, hydrologic conditions are favorable for pathogens, including C-FP-HV to accumulate and cycle within this system (Herbst and Klein, 1995). It has been shown that a related but nononcogenic marine turtle alphaherpesvirus may retain infectivity for several days in seawater (Curry et al., 2000). It has also been suggested that C-FP-HV transmission might be facilitated by ectoparasites (Greenblatt et al., 2004). These potential mechanical vectors could reach high densities in lagoons and bays.

Limited egress of infected turtles from the Indian River system could also limit the dispersion of variant B to other locations. Previous work has suggested that green turtles recruit to the Indian River Lagoon as postpelagic stage juveniles that are several years old and 20–30 cm in carapace length (Ehrhart, 1991; Foley et al., 2005). Turtles captured and marked in the lagoon are rarely recaptured on the Atlantic Ocean side of the barrier island, whereas turtles originally marked on the ocean side within 1 km of an inlet have been recaptured in the lagoon (Ehrhart, 1991). This suggests predominantly unidirectional migration. If turtles acquired C-FP-HV infection before immigration into the Indian River system, one would expect the primary variant to be variant A because this was the predominant form in other Florida locations. Furthermore, FP is uncommon in turtles on the reef outside the lagoon, thus lowering the probability of virus variant A entry into the lagoon (Ehrhart, 1991; Herbst et al., 1998). Thus our data from the Indian River Lagoon system

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strongly suggests that epizootic transmission of variant B occurs during the juvenile phase in this local habitat. The high prevalence of variant B in the Indian River may reflect the restricted movement of turtles within this system. While green turtle movements may be less restricted in other, more open locations, we hypothesize that during epizootics C-FP-HV infection generally occurs when juvenile turtles recruit to specific nearshore locations.

This pattern of infection also may occur in other marine turtle species, such as loggerheads, that also migrate to neritic locations as juveniles. Unfortunately, this species was not represented in our Indian River Lagoon samples. If C-FP-HV is locally transmitted in neritic habitats, loggerhead turtles with FP from the Indian River Lagoon should be predominantly infected by variant B. This possibility warrants additional investigation.

Our observations from Florida provide evidence that within specific locations, an FP outbreak can be linked to a predominant viral variant that is endemic in that habitat. On a regional and global scale, the FP panzootic may represent a number of parallel ongoing outbreaks. Thus, it will be important to characterize the virus variants present in each region. Not only will this better define existing outbreaks, but will provide a means to document the spread of FP to new localities.

The detection of two closely related variants (A and C) in multiple species confirms that interspecies transmission of C-FP-HV variants occurred after each variant had acquired all of its unique nucleotide polymorphisms; this may have occurred within the last few thousand years or in contemporary time (Herbst et al., 2004). However, it is unclear whether cross-species transmission occurs frequently or whether these variants are more likely to segregate independently within each species. That the same virus infects several turtle species in Florida is a serious concern because Florida nearshore waters provide developmental habitat for some highly endangered species, such as the Kemp's ridley. Although the population of nesting adult females of this species is increasing, an epizootic of FP among immature Kemp's ridleys in developmental habitats could significantly affect future recruitment to the breeding population. The conclusion that C-FP-HV is acquired after turtles recruit to nearshore developmental habitats, suggests that it may be possible to improve management of marine turtles by focusing research efforts on understanding and then breaking the cycle of disease transmission in these locations.

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