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SEROLOGICAL ASSOCIATION BETWEEN SPIRORCHIDIASIS, HERPESVIRUS INFECTION, AND FIBROPAPILLOMATOSIS IN GREEN TURTLES FROM FLORIDA

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ABSTRACT: Serodiagnostic tests for detecting green turtle (*Chelonia mydas*) antibody responses were developed to test the strength of association between exposure to spirorchid trematode antigens or herpesvirus antigens and having green turtle fibropapillomatosis (GTFP). Plasma samples from 46 captive-reared green turtles, including paired pre- and 1-yr post-inoculation samples from 12 turtles with experimentally induced GTFP, were found by enzyme-linked immunosorbent assay (ELISA) to be negative for antibodies to adult spirorchid (*Learedius learedi*) antigens. In contrast, all 12 turtles that developed experimentally induced GTFP converted within 1 yr from having negative to positive antibody reactivity to GTFP-associated herpesvirus antigens, whereas the three controls and four turtles that failed to develop tumors remained negative. Plasma samples from 104 free-ranging green turtles from two Florida (USA) coastal feeding grounds with different GTFP prevalences were tested by ELISA for antibodies to *L. learedi* adult antigens; and there was no statistically significant association between antibody prevalence and sampling site. When a low optical density cutoff value (0.15) was used to interpret ELISA results, 98% of the turtles from each site were spirorchid antibody-positive and there was no association between antibody reactivity to spirorchids and GTFP status. When a higher negative cutoff value was used, however, a statistically significant association between antibody reactivity to spirorchids and GTFP-free status was found. These results suggest that spirorchids do not have a role in GTFP pathogenesis. All 20 of the tumor-bearing lagoon turtles had antibodies to herpesvirus antigens whereas only two (10%) of the tumor-free reef turtles had detectable anti-herpesvirus reactivity. The strong association between antibody reactivity to herpesvirus antigens and GTFP status in both captive-reared and free-ranging turtles is consistent with the hypothesis that the transmissible agent that causes GTFP is a herpesvirus.

Key words: *Chelonia mydas*, fibropapillomatosis, green turtle, herpesvirus, plasma antibodies, spirorchidiasis.

INTRODUCTION

Green turtle fibropapillomatosis (GTFP) is a disease of green turtles, (*Chelonia mydas*), characterized by multiple cutaneous fibro-epithelial tumors. First reported in 1938 from Florida (Smith and Coates, 1938), GTFP has since been recognized as a significant threat to green turtle populations around the world (Balazs and Pooley, 1991; Herbst, 1994).

Potential causes of GTFP have been reviewed by Herbst (1994). Two pathogens that have been found within fibropapillomas, a herpesvirus-like agent (Jacobson et al., 1991) and spirorchid trematode eggs (Smith and Coates, 1939), each have been proposed as potential etiologic agents. The GTFP-associated herpesvirus was first reported in two green turtles with spontaneous GTFP (Jacobson et al., 1991) and subsequently found in additional cases of spontaneous disease (Herbst, 1995) and in experimentally induced tumors (Herbst et al., 1995). Green turtle fibropapillomatosis can be transmitted by a chloroform sensitive filterable agent (Herbst et al., 1995; Herbst et al., 1996), supporting the hypothesis that GTFP is caused by an enveloped virus, possibly the GTFP-associated herpesvirus or a retrovirus (Casey et al., 1997).

Although experimental transmission studies have ruled out spirorchid eggs as a

direct cause of GTFP (Herbst, 1994; Herbst et al., 1995), an indirect role for spirorchidiasis in the epizootiology of GTFP remains possible. For example, migrating blood flukes may facilitate infection with the GTFP agent by damaging tissues, especially skin. Cercariae may serve as vectors of the GTFP agent in enzootic areas. Trematodes may facilitate the development of GTFP by severely debilitating their host (Glazebrook et al., 1981; Glazebrook and Campbell, 1990b), so that the immune system cannot eliminate the GTFP agent before tumors develop. Spirorchid egg deposition within tumor vasculature may trigger a chronic inflammatory and immune response that could result in either a continuing hyperplastic response within tumors or eventual tumor rejection. The effect of concurrent parasitic diseases as a cofactor in the pathogenesis of GTFP deserves further investigation.

Serology can provide indirect evidence to support particular hypotheses about the cause of an infectious disease. Green turtles that have developed clinical fibropapillomatosis should have antibody directed against any etiologic agent and there should be an association between exposure to the etiologic agent and clinical disease (tumor status). Previously naive turtles that develop experimentally induced tumors should convert to seropositivity for the GTFP agent(s). Alternatively, seropositivity to incidental pathogens should have no association with tumor status.

This paper describes the development of serodiagnostic tests to test the strength of association between antibodies against spirorchids or herpesvirus and GTFP status in captive-reared and free-ranging green turtles. This study provides evidence that is consistent with the hypothesis that GTFP is caused by a herpesvirus.

MATERIALS AND METHODS

Free-ranging green turtles were netted at two study sites on the Atlantic coast of Florida between 1992 and 1994. These two sites have been monitored since 1982 and 1988 by one of us (L. M. Ehrhart), and represent two distinct near-shore feeding habitats for juvenile green turtles. These sites are in close geographic proximity and have markedly different GTFP prevalences. The Indian River lagoon site (Indian River County, Florida, USA; 27°49′N, 80°26′W) consists of a shallow (1 to 3 m), muddy bottom with drift algae and seagrass beds. The prevalence of GTFP at this site has averaged 50% since monitoring began in 1982 (Ehrhart, 1991; L. M. Ehrhart, unpubl. data). The Wabasso Beach site (27°47′N, 80°24′W) is approximately 1 km due east of the lagoon site and is a Sebellariid worm reef located on sandy bottom in about 2 to 3 m of water. The primary forage at this site was algae. The prevalence of GTFP at this site has been 0% since monitoring began in 1988 (Ehrhart, 1991; L. M. Ehrhart, unpubl. data).

Blood samples (3 to 10 ml) were collected in heparinized syringes from the dorsal cervical sinus (Owens and Ruiz, 1980). Plasma was separated by centrifugation and frozen in aliquots at −20 C and then stored at −70 C.

Forty-eight plasma samples from 47 free-ranging lagoon turtles and 58 plasma samples from 57 reef turtles were collected. Body sizes of these turtles ranged from 26.8 to 73.4 cm straight carapace length (SCL) and the means ± SD did not differ significantly between sites (44.9 ± 9.5 reef and 42.3 ± 8.8 lagoon, Student's t-test (Zar, 1974), $P > 0.10$). None of the reef turtles had GTFP. Twenty (40%) of the lagoon turtles had GTFP.

Plasma was collected from 46 captive-reared turtles that had been raised from eggs in filtered sea water. This included plasma samples collected prior to inoculation from each of 20 turtles used in a GTFP transmission study (Herbst et al., 1995) and matched plasma samples for the 19 survivors, collected approximately 1 yr post-inoculation. Sixteen of these turtles had been inoculated with cell-free GTFP extracts and tumors were induced experimentally in 12 of these turtles (Herbst et al., 1995). The remaining three turtles were uninoculated controls and did not develop tumors. In addition, plasma samples from the four free-ranging turtles used as tumor donors in that transmission study were collected for testing.

Plasma antibody reactivity to herpesvirus inclusions was detected using an immunohistochemical assay. Herpesvirus inclusion positive tissue sections to be used as target antigens were identified by immunohistochemical screening using a herpesvirus-specific turtle antiserum (Herbst, 1995). This antiserum (plasma) was collected from a captive-reared green

turtle that had an experimentally induced fibropapilloma for nearly 1 yr and subsequently developed additional tumors at other cutaneous sites. Plasma from this turtle showed specific binding to intranuclear antigens within foci of ballooning epidermal degeneration corresponding to eosinophilic intranuclear inclusions that were confirmed by electron microscopy to contain herpesvirus particles (Herbst, 1995). Once intranuclear inclusions were identified, at least 20 sections (6 μm) were cut from each paraffin embedded tissue block, mounted on silanized glass slides, and used as antigen substrates for screening plasma samples.

Substrate tissue sections were deparaffinized in three changes of xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was quenched by incubating slides in 3% H_2O_2 for 10 min. Antigenicity was recovered by incubation in an enzyme solution consisting of 0.125% trypsin (Sigma Chemical Co., St. Louis, Missouri, USA) and 0.1% $CaCl_2$ in phosphate buffered saline (PBS) at pH 7.4 for 20 min at 37 C. Slides were washed for 30 min in three changes of PBS and blotted dry. Slides were then flooded with test plasma diluted 1:10 in PBS and incubated overnight at 22 C in a humidified chamber. Positive control slides, using herpesvirus-specific turtle antiserum (diluted 1:50), and negative control slides, using plasma (diluted 1:10) from a healthy captive-reared turtle, were included in the assay. Following incubation with antisera, the sections were washed for 30 min in three changes of PBS, blotted, and incubated with biotinylated secondary antibody solution for 1 hr. The secondary antibody was a combination of biotinylated monoclonal antibodies HL673 (1 μg/ml) and HL857 (1 μg/ml), specific for green turtle immunoglobulin light chain and 7S Immunoglobulin Y (IgY) heavy chain, respectively (Herbst and Klein, 1995a), in PBS containing normal mouse serum (1:20 dilution). The sections were washed for 30 min in three changes of PBS, blotted, and incubated for 1 hr with horseradish peroxidase conjugated strepavidin (Sigma) diluted 1:300 in PBS. After a final wash, the slides were immersed in substrate (3,3′ diaminobenzidine (Sigma), 500 μg/ml in ice cold PBS) and incubated for 2 to 10 min. The color reaction was monitored in control slides. Sections were counter-stained with Harris's hematoxylin, dehydrated, and mounted. Plasma with specific intranuclear immunoreactivity within foci of ballooning degeneration were considered positive (see Fig. 1).

Plasma antobodies to spirorchid trematodes were detected by an enzyme linked immunosorbent assay (ELISA) using adult antigens of either *Learedius* sp. or *Hapalotrema* sp. and

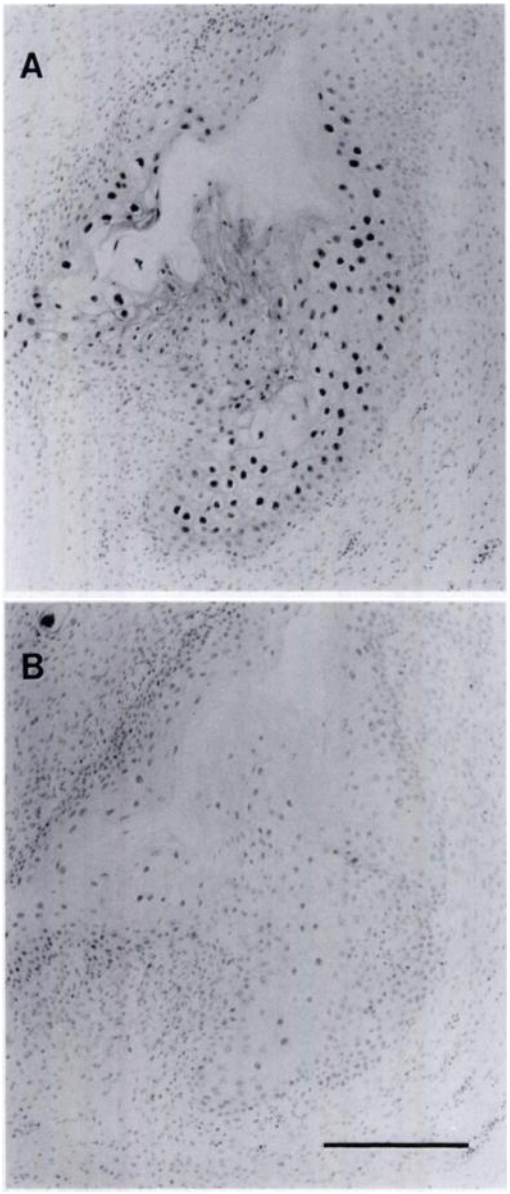

FIGURE 1. Immunohistochemical detection of plasma antibody reactivity to herpesvirus antigens in GTFP tissue sections. (A) Positive intranuclear immunoreactivity confined to epidermal cells undergoing ballooning degenerative changes. (B) Negative immunoreactivity against herpesvirus-positive GTFP tissue section from the same area. Diaminobenzidine substrate counter-stained with Harris' hematoxylin (scale bar = 200 μm).

biotinylated monoclonal antibody HL857, which is specific for green turtle 7S IgY heavy chain. To identify infected and uninfected turtles for use as controls and to collect adult trematodes for use as antigen, post mortem examinations were conducted on sick or injured green turtles that died spontaneously or were euthanized by intravenous pentobarbital overdose (Sleepaway, Fort Dodge Laboratories, Fort Dodge, Iowa, USA). The heart and major blood vessels were opened and examined for cardiovascular flukes. Blood was rinsed through a fine sieve (50 mesh standard) to recover flukes. The gastrointestinal tract and urogenital tract and their contents were examined for helminths. Adult trematodes were collected, identified, and sorted by species. Except for a small specimen taken for histopathology, the spleen was digested in trypsin (0.25% in PBS) for 8 to 12 hr at 37 C. The resulting digest was then centrifuged for 10 min at 500 × G and the pellet resuspended in PBS. This material was layered onto a Percoll (Pharmacia LKB, Uppsala, Sweden) step gradient (80% Percoll stock in PBS) and centrifuged for 30 min at 500 × *g* to separate trematode eggs from tissue debris. Similar digests of fibropapillomas and fibromas collected from those turtles with GTFP also were prepared. Specimens of heart, lung, liver, kidney, spleen, and tumors were fixed in 10% buffered formalin, processed through a graded alcohol series, and embedded in paraffin; 6 μm sections were stained with hematoxylin and eosin and examined for spirorchid eggs by light microscopy.

Two species of adult spirorchid trematodes found at necropsy in three turtles were used to prepare antigens. Forty adult specimens of *Learedius learedi* were homogenized in PBS containing 0.02% sodium azide (PBS/az) using a Tekmar Tissuemizer (Tekmar, Cincinnati, Ohio, USA) and followed by sonication. The resulting extract was filtered through 0.2 μm filter adjusted to a protein concentration of approximately 625 μg/ml and stored in aliquots at −70 C. Antigen from three adult *Hapalotrema* sp. was prepared in the same way.

Optimization experiments were performed using positive and negative control plasma obtained from two adult spirorchid positive turtles and three spirorchid negative turtles, respectively, as determined by necropsy, spleen digest, and histopathology. The *L. learedi* crude antigen stock we prepared was diluted to concentrations ranging from 0.5 to 20 μg/ml in PBS/az and 50 μl of each concentration was applied to 12 wells of an ELISA plate (Maxisorp F96, Nunc, Kamstrup, Denmark). Plates were allowed to incubate overnight at 4 C. The ELISA plates were then washed four times in an automated plate washer with 0.05% Tween 20 (Sigma) in PBS (PBS-Tween). Plates were then blocked for 1 hr at 22 C with 1% BSA in PBS. After washing four times with PBS-Tween, each well with a given antigen coating concentration received positive control plasma diluted from 1:10 through 1:640 in PBS, to form a checker board pattern of antigen and plasma dilution combinations. Negative control plasma was also tested, and 12 wells were used as blank (PBS) controls. After washing, 50 μl biotinylated monoclonal antibody HL857 was applied at 1 μg/ml concentration to each well. After 1 hr incubation, the plates were washed and a 1:1,000 dilution of strepavidin linked alkaline phosphatase (Sigma) was added to each well and incubated for 1 hr. After final washing, 100 μl substrate was added to each well and incubated at 22 C. Optical density at 405 nm was read after 30 and 60 min of incubation in an automated ELISA plate reader. A duplicate plate on which no antigen was coated was also run to determine the amount of non-specific binding in the assay.

Once the optimal antigen coating concentration was determined, known positive and negative control plasma were evaluated out to a 1:1,280 dilution. A similar experiment using *Hapalotrema* sp. antigen was conducted also to test for plasma cross-reactivity. A blocking control plate, omitting the antigen coating step, was also run. Because some samples showed non-specific binding to the control plate (no antigen), even at 1:160 dilution in PBS, these experiments were repeated with samples that were diluted in PBS with NaCl added to a final 0.5 M concentration. The added salt reduces non-specific binding (Herbst and Klein, 1995a).

A protocol was chosen for screening a large number of samples with limited antigen. The protocol used Nunc Maxisorp plates coated with 50 μl per well of adult *Learedius* sp. antigen at 10 μg/ml concentration. This antigen concentration gave the largest difference in optical density readings between positive and negative control plasma. Plasma samples were tested at a 1:10 dilution in 0.5 M NaCl-PBS, and optical density at 405 nm read at 30 min was used in analyses. All plasma samples were assayed at the same time to reduce assay variability. Plates included replicate positive and negative pool sera as well as blank controls. Negative cutoff values for interpreting ELISA results were calculated as the mean plus three standard deviations of OD values from negative control samples.

The strength of association between plasma antibody reactivity to herpesvirus or spirorchid antigens and GTFP status was tested by chi-square analysis (Zar, 1974).

RESULTS

Histologic sections from GTFP biopsies containing enough herpesvirus intranuclear inclusions to use as antigen substrates were uncommon and, therefore, the number of plasma samples that could be tested for antibody reactivity to herpesvirus was limited.

All four GTFP positive turtles used as donors in the transmission study (Herbst et al., 1995) were positive for antibodies to herpesvirus inclusions (Fig. 1A). Seroconversion was associated with the induction of tumors among captive-reared recipient turtles. None of the pre-inoculation plasma samples from these turtles had detectable immunoreactivity to herpesvirus inclusions (Fig. 1B). All 12 turtles that developed GTFP following inoculation with tumor extracts from three separate donors seroconverted within 1 yr post-inoculation. In contrast, the three surviving controls and the four recipients that failed to develop GTFP, after being inoculated with a tumor extract from a single donor, did not seroconvert after 1 yr. The association between immunoreactivity to herpesvirus and positive tumor transmission was statistically significant ($P < 0.001$).

Plasma from the 20 free-ranging GTFP-free turtles from the Wabasso Beach reef were negative ($n = 18$) or very weakly positive ($n = 2$) for antibody reactivity to herpesvirus inclusions. In contrast, all 20 plasma samples from the GTFP-affected turtles from the Indian River lagoon were strongly positive to herpesvirus inclusions. Thus GTFP-free turtles from a low GTFP prevalence site had only a 10% seroprevalence of detectable anti-herpesvirus antibodies while GTFP affected turtles from a high GTFP prevalence site had 100% seroprevalence of anti-herpesvirus antibodies. The association of herpesvirus immunoreactivity with clinical GTFP was statistically significant ($P < 0.001$).

Ante-mortem plasma samples from three captive (tank-reared) turtles and seven free-ranging that were necropsied and examined for spirochid infection were used as controls for the spirorchid antibody ELISA. The three captive (tank-reared) turtles were negative for gastrointestinal helminths, adult cardiovascular trematodes, and their eggs. All seven free-ranging turtles had an abundant and diverse gastrointestinal trematode fauna with species from several families including Pronocephalidae, Paramphistomatidae, Plagiorchidae, Brachycoelidae, Angiodictyidae, Rhytidodidae, Gorgoderidae, Telorchiidae, and Calcoididae. Adult spirorchids were collected from only two of these turtles. One turtle had only two adult *L. learedi* and the second turtle had only six adult *Hapalotrema* sp. Voucher specimens of *L. learedi* and *Hapalotrema* sp. were submitted to the Manter Laboratory, Nebraska State Museum of Natural History, University of Nebraska, Lincoln, Nebraska, USA (accession nos. 38939 and 38940, respectively).

Two types of spirorchid eggs occurred in spleen digests of the free-ranging turtles. Type I eggs were 380 to 460 × 32 to 41 μm, were fusiform with bipolar extensions, and corresponded in morphology to eggs of species of *Learedius* sp. or *Hapalotrema* sp. Type II eggs were ellipsoid and small, measuring 44 to 54 × 37 to 43 μm, and resembled those described for *Neospirorchis* sp. (Wolke et al., 1982; Rand and Wiles, 1985). Only type I eggs were found in the spleen digests of the two free-ranging turtles with adult flukes. Three of the five turtles that were negative for adult flukes had only type II eggs present in their spleens whereas the remaining two had both types of eggs.

Plasma samples from the three captive-reared, helminth negative turtles were used as negative controls in the ELISA. Plasma samples from the two adult spirorchid infected turtles were used as positive controls. The other five turtles that were negative for adult spirorchids and *Learedius* sp.-like type I eggs, but positive for *Neospirorchis* sp.-like type II eggs and gas-

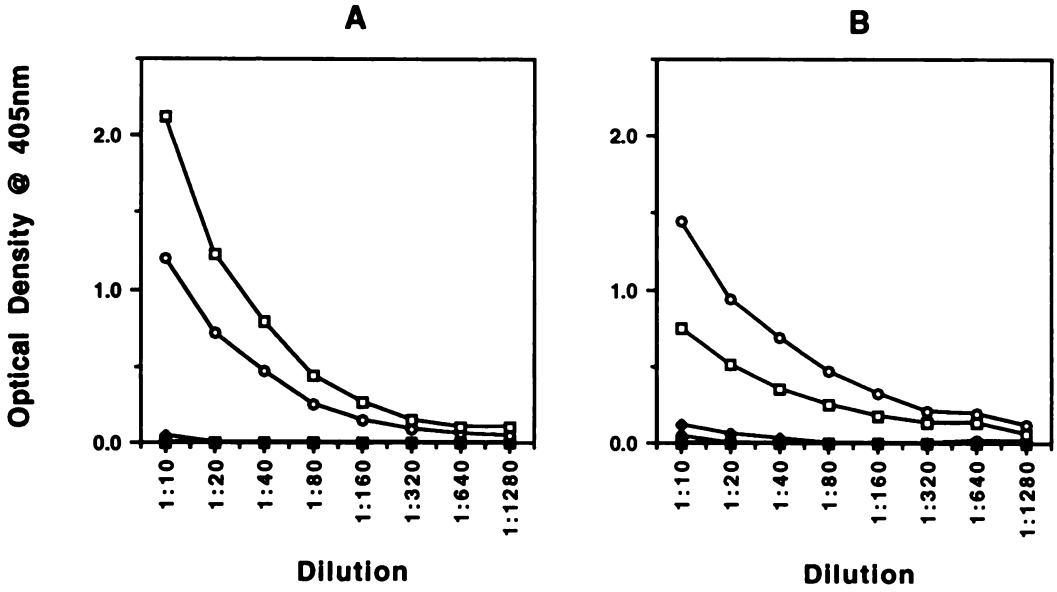

FIGURE 2. Plasma 7S IgY antibody responses of control turtles to crude adult spirorchid antigen preparations. (A) Antibody responses to *Learedius learedi* antigen. (B) Antibody responses to *Hapalotrema* sp. antigen. Positive control samples were from turtles that were positive for either adult *Learedius* sp. (open boxes), or adult *Hapalotrema* sp. (open circles). Negative control samples (solid symbols) were from three captive-reared trematode negative turtles.

trointestinal trematodes were used to help assess the specificity of the ELISA.

Both turtles with active adult spirorchid infections had anti-spirorchid titers, the highest dilution with OD values exceeding the negative cutoff value ($\bar{x}$ + 3SD), between 1:320 and 1:640, using either *Learedius* sp. or *Hapalotrema* sp. crude antigen preparations (Fig. 2A, B). The anti-*Learedius* sp. ELISA results, optical density (OD) values read at 30 min, for plasma diluted 1:10 from the *Learedius* sp. adult positive turtle and the *Hapalotrema* sp. adult positive turtle were 2.36 and 1.11 OD units respectively (Fig. 2A). When the ELISA was run using *Hapalotrema* sp. antigen, similar high OD values were found, but the order was reversed (Fig. 2B). Since, as far as is known, each of these turtles was infected with only a single species, it is apparent that there was considerable antigenic cross-reactivity between these spirorchid species. Thus, either species or both combined could be used as antigen for the screening ELISA. The ELISA protocol for screening plasma samples only used crude antigen from *Learedius* sp. because there was insufficient *Hapalotrema* sp. antigen to test all samples. A plasma dilution of 1:10 for screening was chosen to minimize the chances of false negatives.

Screening ELISA results for the three confirmed negative turtles ranged from 0.028 to 0.087 ($\bar{x}$ ± SD = 0.06 ± 0.03). Using the mean + 3 SD as the cutoff value, samples were considered to be positive if OD > 0.15. The results for 43 captive-reared turtles, including the transmission study turtles, ranged from 0.006 to 0.150 OD units (0.046 ± 0.038) with all values falling within the negative range established with the three proven negative turtles.

The three free-ranging turtles negative for spirorchid adults and type I (*Learedius* sp.-like) eggs but positive for type II (*Neospirorchis* sp.-like) eggs and gastrointestinal trematodes, had OD values ranging from 0.091 to 0.236 (0.167 ± 0.06).

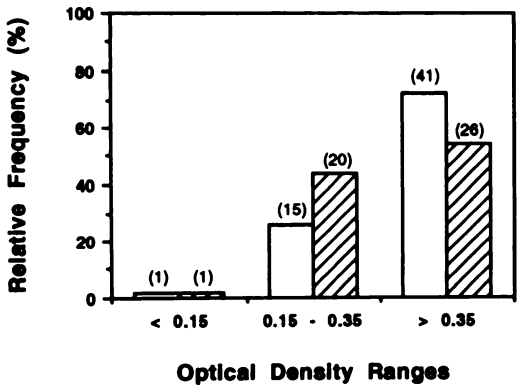

FIGURE 3. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to *Learedius learedi* crude antigen in turtle plasma samples from two sites. The range categories for 30 min readings (OD_{405nm}) correspond to the following: trematode negative (<0.15), adult spirorchid and type I egg negative but type II egg and gastrointestinal trematode positive (0.15 to 0.35), and adult spirorchid or type I egg positive (>0.35). Wabasso Beach reef samples are open boxes; Indian River lagoon samples are cross-hatched boxes. Numbers of turtles in each category are given in parentheses.

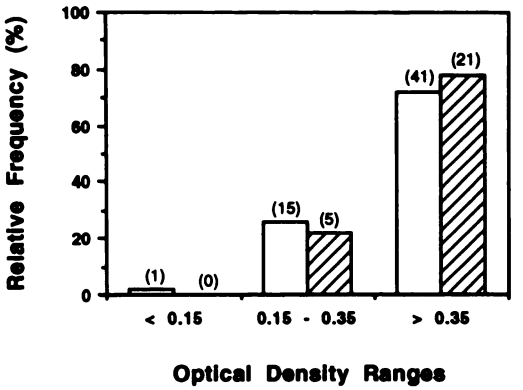

FIGURE 4. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to *Learedius learedi* crude antigen in plasma samples from green turtle fibropapillomatosis (GTFP)-free turtles from two sites. The range categories for 30 min readings (OD_{405nm}) correspond to the following: trematode negative (<0.15), adult spirorchid and type I egg negative but type II egg and gastrointestinal trematode positive (0.15 to 0.35), and adult spirorchid or type I egg positive (>0.35). Wabasso Beach reef turtles are open boxes; GTFP-free Indian River lagoon turtles are cross-hatched boxes. Numbers of turtles in each category are given in parentheses.

Only the lowest OD value was within the negative range. Another plasma sample from this turtle, collected 7 mo earlier, had an OD value above the negative cutoff value. These 3 turtles provided an intermediate OD range with an upper cutoff limit ($\bar{x}$ + 3 SD) of 0.35 for proven type I egg negatives. These low positive OD readings (>0.15 but <0.35) could have been caused by cross-reactions with gastrointestinal trematode species or with spirorchid species, such as *Neospirorchis,* that produce type II eggs. Turtles that were positive for type II eggs but negative for gastrointestinal trematodes, or spirorchid negative but gastrointestinal trematode positive, were not available; thus it was impossible to distinguish between these two possibilities by ELISA. Among the four proven *Learedius* sp.-like egg positive turtles, the lowest OD value was 0.476 which was above (1.4 times) the cutoff limit (0.35) for type I egg negative turtles.

We evaluated the two population samples, Indian River lagoon and Wabasso Beach reef, across the three optical density range categories, for 1:10 diluted plasma (Fig. 3). Only one turtle from each location had an OD value <0.15 and, using this value as the negative cutoff, seroprevalence estimates for spirorchid infection were 98% for both sites. The seronegative lagoon turtle had GTFP and obviously, there was no etiologic association between spirorchid exposure, as measured, and GTFP prevalence. Using the higher negative cutoff value of 0.35, which was more specific for detecting anti-*Learedius* sp. antibodies, the lagoon sample had a lower seroprevalence with 26 (56%) positives than the reef with 41 (72%). However, the association of spirorchid seroprevalence with habitat was not significant ($P > 0.05$).

After exclusion of turtles with GTFP from the habitat comparison (Fig. 4), seroprevalence estimates for spirorchid infections, using the low cutoff value were 100% for tumor-free lagoon turtles and 96% for reef turtles. When the higher cutoff value was used, more tumor-free lagoon turtles were spirorchid-positive com-

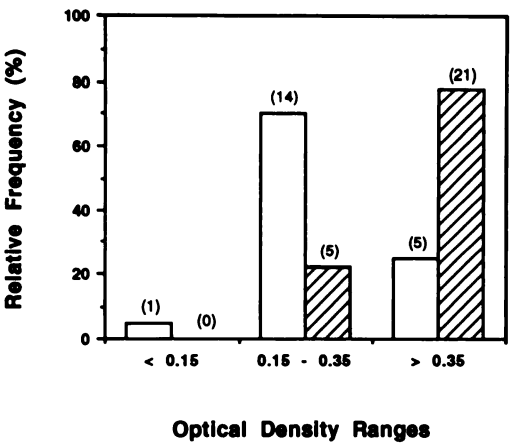

FIGURE 5. Relative frequencies of negative, intermediate, and positive 7S IgY antibody responses to *Learedius learedi* crude antigen in plasma samples from GTFP-positive turtles and GTFP-negative Indian River lagoon turtles. The range categories for 30 min readings (OD_{405nm}) correspond to the following: trematode negative (<0.15), adult spirorchid and type I egg negative but type II egg and gastrointestinal trematode positive (0.15 to 0.35), and adult spirorchid or type I egg positive (>0.35). GTFP-positive turtles are open boxes; GTFP-negative turtles are cross-hatched boxes. Numbers of turtles in each category are given in parentheses.

pared to reef turtles (78% and 72%, respectively) but this difference was not significant ($P > 0.50$). In contrast, there was a significant ($P < 0.001$) difference between the anti-spirorchid antibody seroprevalence of tumor-bearing lagoon turtles (25%) and that of normal tumor-free reef turtles (72%) (data not shown).

Comparing GTFP positive turtles with tumor-free turtles within the lagoon, using the high cutoff value (OD > 0.35), only five (25%) of the 20 GTFP positive turtles were spirorchid ELISA positive, whereas 21 (78%) of the 27 tumor-free turtles were seropositive (Fig. 5). Only five (19%) of the 26 spirorchid ELISA positive lagoon turtles were GTFP positive, whereas 15 (71%) of 21 spirorchid negative lagoon turtles also had GTFP. The association between antibody reactivity against spirorchids, as measured, and tumor-free status within the lagoon sample was significant ($P < 0.001$). These results do not support a positive association between spirorchidiasis and GTFP.

DISCUSSION

The strong association between conversion to herpesvirus antibody reactivity and the development of experimentally induced tumors is consistent with the hypothesis that the GTFP-associated herpesvirus is the etiologic agent of GTFP; however, these results do not prove causation. It is possible that the herpesvirus was cotransmitted along with the true GTFP-agent as a contaminant of the filtered tumor homogenates.

The strong association of detectable anti-herpesvirus antibodies with tumor status in free-ranging green turtles, including the four transmission study donors, also is consistent with a causal relationship between herpesvirus and GTFP. Among free-ranging turtles, there was no possibility of nosocomial herpesvirus infection. Detection of herpesvirus antibodies in free-ranging green turtles from other geographic sites with high GTFP prevalence further supports this hypothesis (Herbst, unpubl. data). On the other hand, it is also possible that the tumor provides a favorable physiological environment for reactivation of a latent herpesvirus infection that is accompanied by a rise in anti-herpesvirus antibody titer. Only successful isolation of this virus and fulfillment of Koch's postulates through transmission studies will prove whether the GTFP-associated herpesvirus causes this disease.

Determination of immunohistochemical reactivity to herpesvirus inclusions was qualitative and somewhat subjective. The specificity of this test for the GTFP-associated herpesvirus relative to other herpesviruses that may infect green turtles has not been determined. Two other herpesviruses have been reported in farm-reared green turtles (Rebell et al., 1975; Jacobson et al., 1986) and, although comparisons of nucleotide sequences in two conserved gene regions showed that the GTFP-associated herpesvirus is distinct from at

least one of these (Herbst et al., 1998), it is possible that these viruses share homologous antigens that cross-react in serodiagnostic assays. A more sensitive, specific, and objective diagnostic test is needed before more extensive population surveys are conducted. Further development of diagnostic tests, such as an ELISA, for the GTFP-associated herpesvirus or other viral agents has been hampered by the limited availability of antigens. Successful isolation of this virus in culture or production of recombinant viral antigens would solve this problem.

The occurrence of spirorchid eggs within fibropapillomas was first noted by Smith and Coates (1939) and was subsequently confirmed by others (reviewed in Herbst, 1994). The natural history of spirorchid trematodes is very similar to that of *Schistosoma* spp. in that adult trematodes inhabit the vascular system and eggs must migrate through tissues to reach the environment (Lauckner, 1985). *Schistosoma mansoni* egg antigens can elicit a fibrotic response in the host (Wyler, 1983; Lammie et al., 1986) leading to the hypothesis that spirorchid eggs may induce GTFP by similar mechanisms (Harshbarger, 1984). However, eggs and lesions associated with eggs have not been found in turtles with experimentally induced GTFP (Herbst et al., 1995) and attempts to produce GTFP by injecting turtles with eggs have failed (Herbst, 1994; Dailey and Morris, 1995). In addition, spirorchid eggs have been found in free-ranging, oceanarium-reared, and farmed green turtles from Queensland, Australia where the prevalence of fibropapillomatosis was 0% (Glazebrook and Campbell, 1990a, b).

In contrast to the herpesvirus serology results, the association between immunoreactivity to spirorchid antigens and clinical GTFP was either weak or strongly negative, depending on how the ELISA values were interpreted. Either way, the data do not support any hypotheses that involve spirorchidiasis in the epizootiology or pathogenesis of GTFP. When all samples with OD values above the cutoff value (0.15) for trematode negative turtles were interpreted as positive for spirorchid exposure, assuming no false positives caused by cross-reactions with gastrointestinal species, nearly all (98%) juvenile turtles were positive; consequently, there was no statistical association between spirorchid exposure and GTFP prevalence.

When the higher cutoff limit (0.35) was used, assuming OD values between 0.15 and 0.35 were false positives, then seroprevalence estimates ranged from 25% for GTFP-positive lagoon turtles to 78% for tumor-free lagoon turtles. Although there was a statistically significant association between GTFP status and spirorchid antibody reactivity, the relationship was the opposite of that predicted by the hypothesis that tumors are caused by spirorchid eggs (Harshbarger, 1984).

Results obtained by using the higher cutoff value (0.35) could be interpreted in several ways. Immunosuppression, causing a decline in spirorchid-specific humoral immune responses could explain the lower prevalence of high OD positive ELISA responses in GTFP-affected turtles. However, one of the adult positive turtles and two type I spirorchid egg positive turtles had severe, debilitating fibropapillomatosis and yet had very high OD values. In addition, all GTFP-positive turtles had detectable antibodies to the GTFP-associated herpesvirus. Immunomodulation, following tumor induction, resulting in down-regulation of anti-spirorchid responses and up-regulation of anti-GTFP agent responses, could also explain these results. Another explanation is that the lagoon habitat does not support an active spirorchid life cycle, so that turtles moving into the lagoon would no longer become reinfected. Anti-spirorchid antibody titers would decline with time as resident turtles eliminated any infections they brought with them into the lagoon. Prolonged residency in the lagoon also would increase the probability of contracting GTFP if the lagoon was the reservoir for the infectious

GTFP agent and associated cofactors (Ehrhart, 1991; Herbst and Klein, 1995b). This hypothesis could be tested by comparing plasma samples from long-term lagoon residents with those from turtles that recently entered the system, or by analyzing a series of samples from turtles held in pens within the lagoon. A serologic survey of green turtles from other lagoon habitats with lower GTFP prevalences may also help resolve this question.

If the lagoon habitat does not support completion of spirorchid life cycles then it becomes important to determine where and when green turtles become infected. Spirorchid infections may be acquired by very young green turtles in the pelagic environment during their 2 to 5 yr pelagic existence. Small green turtles (25 to 30 cm SCL) are likely to be recent migrants from the pelagic environment (Bolten and Bjorndal, 1992) and turtles in this size range have been documented with spirorchidiasis (Looss, 1902, cited in Lauckner, 1985). The smallest turtle in this study (26.8 cm SCL) had antibodies to spirorchids. Sampling of pelagic juvenile green turtles will help resolve this question.

The ELISA described in this report was designed to detect 7S IgY antibodies to adult *L. learedi* crude antigen and provided a rapid non-destructive method to estimate the prevalence of spirorchid infection in turtle populations. However, there were limitations to data interpretation because of potential cross-reactivity with irrelevant antigens. On one hand, cross-reactivity was useful because, although *L. learedi* is the most commonly reported species in green turtles from Florida waters and in the Caribbean (Greiner et al., 1980; Rand and Wiles, 1985; Dyer et al., 1991), it is not the only species found in fibropapillomas. The ELISA was able to detect antibody reactivity in plasma from a turtle infected with *Hapalotrema* sp. adults only, in plasma from two turtles whose tissues contained characteristic *Learedius* sp.-like eggs, and in plasma from three turtles whose tissues contained only Type II (*Neospirorchis* sp.-like) eggs. Thus, the ELISA was sensitive enough to detect exposure to spirorchids where there were too few flukes to detect at necropsy or where the duration of exposure and time since clearance of infection were unknown. In addition, the screening protocol was able to correctly classify plasma from 43 captive-reared and presumed negative turtles as negative (0% false positives).

On the other hand, potential cross-reactivity with gastrointestinal trematodes made it difficult to interpret OD values between 0.15 and 0.35. The three control turtles with ELISA values within this range had been infected with type II egg producing spirorchids and gastrointestinal species. Because samples from turtles diagnosed with only one type of infection (type II spirorchid or gastrointestinal species) were not available, it remains unclear whether these values were diagnostic for spirorchidiasis. Similar problems with serologic cross reactivity between gastrointestinal trematodes and *Schistosoma* sp. have been reported in mammals (Derouin et al., 1980; Hillyer and Sagramoso de Ateca, 1980).

The ELISA appeared to be sensitive and specific for antibodies to *Learedius* sp. and *Hapalotrema* sp. (type I egg producing species) when the higher cutoff value (0.35) was used, since all four samples from turtles with proven exposure to type I species had OD values well above this limit. Additional plasma samples from turtles with well documented trematode exposures will be needed to evaluate this ELISA further. Studies are needed to identify antigens that are unique to spirorchid species for development of more refined serologic tests. Also, because antigenic cross-reactivity may vary among spirorchid species, a battery of ELISA's, each using antigen from a different species, should be used for detection of green turtle antibody responses to spirorchidiasis.

In conclusion, the strong association between antibody reactivity to herpesvirus

antigens and clinical GTFP was consistent with the hypothesis that the GTFP-associated herpesvirus, first reported by Jacobson et al. (1991) and subsequently observed in experimentally induced tumors, as well as additional spontaneous tumors (Herbst, 1995; Herbst et al., 1995), is the cause of GTFP. While there is no doubt that spirorchid trematodes are important pathogens of green turtles and that concurrent spirorchidiasis may influence the clinical course of GTFP, the serologic data presented here support earlier opinions (Smith and Coates, 1939) and experimental evidence (Dailey and Morris, 1995; Herbst et al., 1995) that the occurrence of spirorchid eggs in tumors is an incidental finding. A clearer understanding of the epizootiology of herpesvirus and spirorchid infections in green turtle populations must await further studies with improved serodiagnostic assays.

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