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DETECTION BY ELISA OF CIRCULATING ANTI-BLOOD FLUKE (*CARETTACOLA, HAPALOTREMA,* AND *LEAREDIUS*) IMMUNOGLOBULINS IN HAWAIIAN GREEN TURTLES (*CHELONIA MYDAS*)

Thaddeus K. Graczyk, A. Alonso Aguirre*, and George H. Balazs†
Department of Molecular Microbiology and Immunology, School of Hygiene and Public Health, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205

ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) is described utilizing the surface glycocalyx crude antigen of adult blood trematodes *Learedius learedi, Hapalotrema dorsopora,* and *Carettacola hawaiiensis,* for the detection of circulating antibodies (Ab) in Hawaiian green turtles (*Chelonia mydas*) naturally infected with the parasites and with or without green turtle fibropapillomatosis (GTFP). A concentration of 10.0 μg/ml of antigen was optimal in terms of test specificity and sensitivity. A direct ELISA with anti-reptilian/amphibian phosphatase-labeled IgG identified *C. mydas* Ab at a dilution of 1/12,800. Utilizing indirect ELISA, it was possible to detect Ab to blood flukes at a dilution of 1/3,200. The gross lesions and histopathology were typical for cardiovascular spirorchidiasis in *C. mydas.* No significant relationship was found between the size of turtles and the degree of GTFP severity. Forty-seven of 59 (80%) samples, from 5 sites, gave a positive ELISA reaction; 6 of the 47 (13%) specimens gave significantly ($P < 0.001$) higher absorbance values, with 5 from the same location. All 12 (20%) ELISA-negative turtles originated from 1 site, and the absorbance values of the 41 animals from this location were significantly lower ($P < 0.015$) when compared with the other 4 sites. The proposed assay is fast, has the feature of visual scoring, and can be used for determination of exposure of *C. mydas* to the spirorchid trematodes.

The Hawaiian green turtle (*Chelonia mydas*) is a geographically isolated species protected under the United States Endangered Species Act and Wildlife Laws of the State of Hawaii. Although the population has shown a gradual increase since 1978, *C. mydas* has demonstrated a slow rate of recovery (Balazs, 1991). Cardiovascular spirorchidiasis (Digenea: Spirorchidae) caused by *Learedius learedi, Hapalotrema dorsopora,* and *Carettacola hawaiiensis* can affect the survival of *C. mydas* (see Dailey et al., 1992; Aguirre, 1994; Aguirre and Spraker, 1994). Cardiovascular Spirorchidae exhibit a worldwide distribution (Platt, 1992) and are frequently found in *C. mydas* (Greiner et al., 1980; Glazebrook et al., 1989; Dailey et al., 1993; Aguirre and Spraker, 1994; Aguirre, Balazs, Zimmerman, and Spraker, 1994). The life cycle of these spirorchids has not been elucidated. Studies on the cercariae in snails from infected turtle habitats yielded negative results (Greiner et al., 1980; Dailey et al., 1992), indicating that the life cycle described for spirorchids from freshwater turtles (Smith, 1972) may not apply for *C. mydas.*

Green turtle fibropapillomatosis (GTFP) is a debilitating and disfiguring neoplastic condition increasing in geographic range and magnitude. A viral agent has been suggested as the primary etiological agent (Jacobson et al., 1989; Balazs and Pooley, 1991). Other etiologic factors include a response to trematode ova (Dailey et al., 1992), environmental pollutants impairing the immune system (Aguirre, Balazs, Zimmerman, and Galey, 1994), chronic stress (Aguirre, 1994), and a genetic predisposition to neoplasia (Balazs and Pooley, 1991). Co-occurrence of spirorchidiasis and GTFP causes debilitation and fatality in juveniles of *C. mydas* in Hawaii (Aguirre and Spraker, 1994).

The prevalence of spirorchid infections of *C. mydas* in Hawaii has been based on field necropsies; however, this method was utilized selectively (Dailey et al., 1992; Aguirre and Spraker, 1994). Only turtles that were found dead or unable to survive in their natural habitat were necropsied. Considering the threatened status of *C. mydas* in Hawaii, necropsy of clinically healthy individuals cannot be used as a technique for helminthological surveys. A diagnostic test to determine blood trematode infection in green turtles is not available, and the incomplete knowledge of pathogen transmission prevents the development of programs for the control and prevention of the infection. The purpose of the present study was to develop an enzyme-linked immunosorbent assay (ELISA) for detecting circulating anti-blood fluke immunoglobulins in Hawaiian green turtles and to determine the prevalence of parasite infection within a discrete population of *C. mydas.*

MATERIALS AND METHODS

Fifty-eight wild and 3 captive Hawaiian green turtles (*C. mydas*), aged 5–25 yr old, of unknown sex, were used. During the month of September (1991–1993), 46 wild turtles were captured while snorkeling at 3 sites in Kaneohe Bay, Island of Oahu; Ahu-O-Laka (n = 41), Mark Reef (n = 3), and Reef 42 (n = 2). In September 1993, 10 wild turtles were collected with large-mesh tangle nets in Keawanui Bay at the Island of Hawaii. Two wild turtles, severely affected by GTFP, were recovered through the National Marine Fisheries Service (NMFS) Hawaiian Sea Turtle Stranding and Salvage Program in Haleiwa and Kailua Bay, at the Island of Oahu. Captive *C. mydas* collections included 1 turtle kept for 5 yr at the NMFS research facilities due to traumatic amputation of front flippers, and 2 captive-hatched *C. mydas* from Sea Life Park at Island of Oahu, and raised in an Aquarium in Seattle (Washington). The straight carapace length (SCL) (cm) and weight (WGT) (kg) of each animal were recorded in research facilities of the Hawaii Institute of Marine Biology at Coconut Island, Kaneohe Bay, Oahu. Tumors were scored by degree of GTFP severity on a scale of 0–4, with 4 being the most severe case (Balazs, 1991). Blood (5–10 ml) was collected in a 10-ml heparinized syringe by venipuncture from the bilateral dorsal cervical sinuses (Owens and Ruitz, 1980). The sample was placed in heparinized Vacutainer tubes, centrifuged (2,000 *g,* 10 min), and plasma stored at −70 C.

A moribund turtle, severely affected with GTFP was euthanized with a lethal intraperitoneal injection of Beuthanasia-D Special Solution (Schering-Plough Animal Health Corp., Kenilworth, New Jersey), exsanguinated, and necropsied according to the protocol of Wolke et al.

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* P.O. Box 1522, Fort Collins, Colorado 80522.
† National Marine Fisheries Service, Southwest Fisheries Science Center, Honolulu Laboratory, 2570 Dole Street, Honolulu, Hawaii 96822-2396.

(1982). The blood was centrifuged (10,000 *g*, 15 min), and the plasma was stored in 10–20-ml aliquots at −70 C. Lungs, liver, heart and adjacent vessels, and mesenteric arteries were examined for trematodes. Adult worms were placed in a petri dish with phosphate-buffered saline (PBS) (pH 7.4), counted, identified to species under a dissecting microscope, removed from PBS, and stored at −70 C. The identification of the specimens followed the descriptions by Skrjabin (1964) and Dailey et al. (1991, 1993). Organ sections were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (Aguirre and Spraker, 1994).

For the antigen preparation, the flukes were thawed, pooled, and each of 5 pools (approximately 25 worms per pool) was suspended in 1.0 ml of PBS (pH 7.4) to which 1.0 ml of 85% phenol was added. The incubation, centrifugation, and dialyzing procedures followed the protocols of Caufield et al. (1987) and Nanduri et al. (1991). The concentration (0.89 mg/ml) of the glycocalyx-containing fraction was determined by a Milton Roy Spectronic 1001 spectrophotometer (Milton Roy, Durham, North Carolina) at a 280-nm wavelength. The final preparation was diluted 1/10 with PBS, stored at −70 C in 1.5-ml vials, and then thawed and aliquoted prior to the ELISA trials.

A direct ELISA was performed to determine the binding efficacy of anti-reptilian/amphibian IgG conjugated to alkaline phosphatase to immunoglobulins in the plasma of *C. mydas*. Anti-reptile/amphibian serum was generated in New Zealand white rabbits (Irby and Apperson, 1988). Purification of anti-reptilian/amphibian IgG from the serum by a protein A column (low salt) chromatography, and coupling to alkaline phosphatase (type VII-T) (Sigma Chemical Co., St. Louis, Missouri) followed the protocol of Graczyk et al. (1994). Optimum signal with low background was achieved using Immulon 2 microtitration plates (Dynatech Laboratories Inc., Chantilly, Virginia). The tested plasma was prepared by pooling of 0.25 ml of plasma from each of 59 samples. Eight wells on an ELISA plate were filled with 100 μl of the following PBS dilutions of turtle plasma pool: 1/50, 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400, and 1/12,800. A negative control (NC) for this trial was prepared by coagulation of immunoglobulins and immunocomplexes from the pool (Johnson et al., 1989). The plate was covered and incubated overnight at room temperature (RT). Each well was then washed twice with PBS/0.05% Tween-20 (TW-20). One hundred microliters of anti-reptilian/amphibian IgG, conjugated to alkaline phosphatase diluted (1/500) with boiled 0.5% casein blocking buffer (CBB), was added to each well. The plate was incubated at 37 C. After 1 hr, the wells were washed 3 times with PBS/TW-20 and filled with 100 μl of 1.0-mg/ml *p*-nitrophenyl phosphate (*p*-NPP) substrate in 0.1 M glycine buffer (GB). The enzymatic reaction was stopped as described previously (Graczyk et al., 1993).

In the indirect ELISA, the plate was coated with 100 μl of antigen (10.0 μg/ml) and incubated overnight at RT. Eight wells on the plate were not coated in order to determine nonspecific background values (NBV) of absorbance. Following coating, the plate was postcoated by incubating each well for 3 hr at RT with 200 μl of CBB/0.05% TW-20. Following postcoating, the wells were then emptied and filled in triplicate with 100 μl of test plasma diluted (1/50) with PBS (pH 7.4). Negative control was a pool of the plasma obtained from the 2 green turtles from Sea Life Park at Island of Oahu, Hawaii. The animals were hatched in captivity and were raised in an aquarium in Seattle, Washington. These turtles were kept indoors under parasite-free conditions and fed on a diet of human-food-grade squid. The positive control (PC) was prepared by dilution (1/50 with PBS, pH 7.4) of the plasma of *C. mydas* from which the trematodes were recovered. Negative controls and PC were prepared at the same time in several 1.0-ml vials, frozen at −70 C, and a single sample was thawed for the ELISA, then discarded after use. This procedure prevented changes in the background caused by repeated freeze–thaw cycles. Eight wells were filled with NC, and 8 wells were filled with PC. After 3 hr incubation at 37 C, the plates were washed 6 times with PBS/0.05% TW-20. One hundred microliters of anti-reptile/amphibian IgG coupled to alkaline phosphatase diluted in PBS (pH 7.4) (1/500) was added to each well, and the plate was processed as described above. The reproducibility test for the indirect ELISA was performed 2 wk later with the same 59 plasma samples following the same protocol.

To determine the lower limits of the indirect ELISA, 8 wells were filled with 100 μl of the following PBS dilutions of PC plasma: 1/50, 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400, and 1/12,800. The test was run according to the indirect ELISA protocol. The coded trial to determine the test reliability was performed with 42 serum samples. Eight serum samples were prepared from the 4 NC plasmas that were used in the indirect ELISA, and 34 samples were randomly drawn from the 59 samples tested before. The sera were coded and the test was performed by 1 of us (T.K.G.) without knowledge of the identity of the samples.

To determine the specificity of the indirect ELISA, the plate was coated with 10.0 μg/ml of glycocalyx antigen from the intestinal trematode *Echinostoma caproni* (Graczyk and Fried, 1994), obtained in the same manner as from *H. dorsopora, L. learedi,* and *C. hawaiiensis*. The use of mouse NC and PC followed the protocol of Graczyk and Fried (1994). Indirect ELISA with 59 *C. mydas* plasma samples was performed as described above.

The absorbances were read (405 nm) on a MAXline microplate reader controlled by a computer program (SOFTmax, Molecular Devices Corp., Menlo Park, California), which subtracted the NBV from all values on the plate, giving the corrected absorbance values used for analysis. The positive cutoff level was established as units of antibody (Ab) greater than the mean of absorbance of NC ± 3 SD. The adjustment method of Schwartz et al. (1991) was used to compare absorbance values from different ELISA plates. The absorbance values were comparable from plate to plate because the same NC and PC was used in each plate. The mean adjustment from plate to plate was 1.86%, and the results did not differ when unadjusted absorbances were used for analysis. Adjusted absorbance values are presented for comparison purposes.

Statistical analysis was performed with Analytical Software Statistix 3.5 Program (Analytical Software, St. Paul, Minnesota). Analysis of variance (ANOVA) was used to determine significance of the among-turtle-group effect. A 2-sample *t*-test was performed to determine the statistical significance between the means from different ELISA plates, a paired *t*-test was used for the means obtained from the same plates, and a *G*-test was used to compare the percentage of positive or negative animals among the groups. The degree of linear association between variables was compared using Pearson's correlation coefficient (*r*). Significance was considered to be $P < 0.05$. A nonparametric Wilcoxon signed rank test was used to examine relationships among the GTFP tumor severity score, size, and absorbance values. Other statistical treatments followed the procedures of Sokal and Rohlf (1981).

RESULTS

A total of 78 *H. dorsopora* and 34 *L. learedi* was found in the heart chambers and major vessels, and 17 *C. hawaiiensis* were recovered from the hepatic vessels of the euthanized turtle. Gross lesions included variously sized, lobulated tumors, serous atrophy of fat, and edema in subcutaneous tissues, pectoral, and coracoid muscles. Hydropericardium, pulmonary edema, a generalized thickening and hardening of major vessels with partial or complete obliteration of blood flow, and thrombi formation were observed. Histologically, a generalized lymphoplasmacytic endarteritis and thrombosis, and multifocal granulomas associated with trematode ova were observed throughout the parenchyma of the skin salt glands, heart, lungs, gastrointestinal tract, spleen, and kidneys. The granulomas were composed of multinucleated giant cells and macrophages engulfing parasitic ova.

The SCL of the 59 turtles was significantly correlated with the WGT of the animals ($r = 0.839$, $P < 0.001$); however, the Wilcoxon signed rank test did not demonstrate a significant relationship between SCL (or WGT) and the tumor severity score ($P > 0.05$).

In preliminary experiments, various dilutions of the crude surface glycocalyx antigen of *L. learedi, H. dorsopora,* and *C. hawaiiensis* were used for the coating of ELISA plates. A concentration of 10.0 μg/ml of antigen was optimal in terms of test signal-to-noise ratio. Using the protocol for the indirect ELISA,

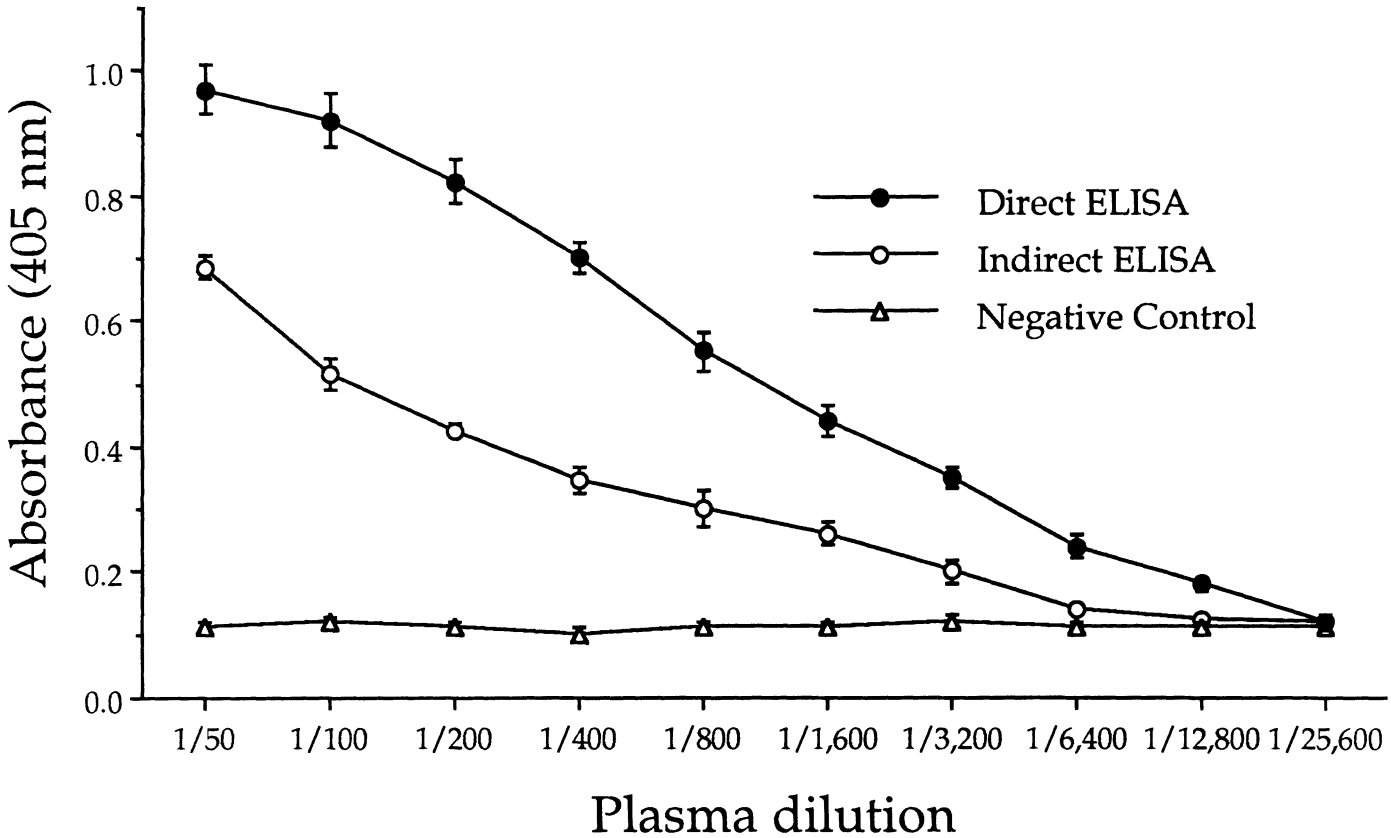

FIGURE 1. Binding efficacy of alkaline phosphatase anti-reptilian/amphibian IgG and immunoglobulins in the plasma of green turtles (*Chelonia mydas*) in a direct enzyme-linked immunosorbent assay (ELISA), and indirect ELISA detection with surface glycocalyx crude antigen of adult *Learedius learedi, Hapalotrema dorsopora,* and *Carettacola hawaiiensis* of anti-blood fluke immunoglobulins in the plasma of naturally infected turtle. Negative control (NC) plasma for indirect ELISA originating from uninfected captive *C. mydas*; NC for direct ELISA was obtained by coagulation of immunoglobulins and immunocomplexes from plasma by heating (95 C) with citric acid. Each symbol represents the mean of absorbance of 8 ELISA wells ± SD.

a low NBV of absorbance (less than 0.074) was observed. The enzymatic reaction was done routinely for 30 min; extension of incubation with substrate (*p*-NPP) to 40, 60, and 120 min resulted in an increase NBV of absorbance to 0.084, 0.089, and 0.097, respectively.

The positive absorbance values in the direct ELISA ranged from 0.97 to 0.23, $\bar{x} = 0.58 \pm 0.29$ (Fig. 1) and regressed significantly ($r = 0.73$, $P < 0.002$) with dilutions of the 100-μl turtle plasma. The absorbance values for the dilutions of 1/50–1/12,800 did not reach the cutoff level (0.16) and were statistically significant by paired *t*-test ($t = 4.76$, $P < 0.002$) when compared with the NC absorbance.

The range of absorbance values for PC in the indirect *C. mydas* ELISA was 0.70–0.67 ($\bar{x} = 0.68 \pm 0.01$) and 0.11–0.12 ($\bar{x} = 0.11 \pm 0.01$) for the NC, with the cutoff level of 0.14. The ELISA results showed that 47 of 59 (80%) samples gave positive reaction with crude surface glycocalyx antigen. The positive absorbance values were not significantly correlated with the size of *C. mydas* ($r = 0.146$, $P = 0.328$) and were not significantly (Wilcoxon signed rank test; $P > 0.05$) related to the GTFP tumor severity score. Six of 47 (13%) samples gave significantly (ANOVA; $F = 89.7$, $P < 0.001$) higher absorbance values (range: 0.674–0.614, $\bar{x} = 0.646 \pm 0.02$) than the rest of the positive samples (range: 0.550–0.311, $\bar{x} = 0.41 \pm 0.06$); 5 of 6 of these samples originated from the same site (Keawanui Bay at the Island of Hawaii) (Table I). The absorbance of 12 of 59 (20%) samples did not reach the cutoff level; these 12 negative turtles originated from 1 site, Ahu-O-Laka at Kaneohe Bay on the Island of Oahu (Table I). When the negative turtles were extracted from statistical analysis, ANOVA showed that the absorbance values of the animals from Kaneohe Bay on the Island of Oahu (Table I) were significantly lower ($F = 6.44$, $P < 0.015$) than from the Keawanui Bay at the Island of Hawaii. The reproducibility indirect ELISA trial identified the same 13 samples as negative and the same 47 samples as positive.

The range of absorbance values for PC in the indirect ELISA with *E. caproni* antigen was 0.89–0.91, with a mean of 0.90 ± 0.01; however, all *C. mydas* plasma samples gave a negative reaction.

Using rabbit anti-reptile/amphibian alkaline phosphatase-labeled IgG, it was possible to detect turtle immunoglobulins up to a dilution of 1/3,200 (Fig. 1). The differences between the NC from the indirect ELISA and direct ELISA were not significant (2-sample *t*-test; $t = 1.78$, $P = 0.139$).

The ELISA with coded turtle plasma showed that absorbances of 11 of 42 animals were not significantly different (paired *t*-test; $t = 1.18$, $P = 0.405$) from the absorbance of the NC. After revealing the status of the negative specimens, it appears that they included 8 NC and 3 samples determined to be negative by the previous indirect ELISA. The absorbance values of the

remaining 31 samples were significantly (paired t-test; $t = 155.75$, $P < 0.001$) higher than the absorbance of NC, and it appears that all originated from positive animals.

DISCUSSION

Gross pathology and histopathology of the *C. mydas* from which the flukes were recovered confirmed the pathological findings associated with concurrent spirorchidiasis and GTFP (Aguirre and Spraker, 1994; Aguirre, Balazs, Zimmerman, and Spraker, 1994). The site of infection of *C. hawaiiensis* confirmed the observation of Dailey et al. (1991) on the exclusive occurrence of this trematode species in hepatic vessels of marine turtles. The histopathological changes related to tissue granulomas caused by *Learedius* sp., *Carettacola* sp., and *Hapalotrema* spp. in *C. mydas* from Australia have been described by Glazebrook et al. (1989). The histopathology and pathogenesis of parasitic granulomas associated with chronic vasculitis in loggerhead turtles (*Caretta caretta*) from the Atlantic seaboard were described by Wolke et al. (1982).

Parasitological surveys in Hawaiian *C. mydas* with GTFP demonstrated prevalences of 80–100% of *H. dorsopora,* 40% of *L. learedi,* and 30% of *C. hawaiiensis* (Dailey et al., 1992, 1993). The distribution of infection with these trematodes was not affected by turtle size, sex, or turtle origin (Dailey et al., 1992). In the present study, however, the differences in ELISA-Ab titer among various groups of animals were significantly related to the geographic origin of the turtles.

The weakness of the necropsy-derived data is the selective targeting of stranded turtles unable to survive in the wild. This is misleading for the estimation of real values of prevalence of infection in a wild population of *C. mydas.* As shown by Glazebrook et al. (1989), necropsy-negative turtles revealed the presence of egg granulomas during histologic examination. In this context, the ELISA may support the routine monitoring of discrete *C. mydas* populations in Hawaii by determining their exposure to spirorchid trematodes. Monitoring the Ab titer over time in selected marked turtles may reveal epidemiological insights on when and where trematode infections are acquired and their relationship to GTFP.

Jacobson et al. (1989) examined histological biopsies of GTFP in *C. mydas* from Hawaii and from the Indian River Lagoon System in Florida. They found trematode ova only in the biopsies from the Hawaiian population. Aguirre (1994) studied cellular responses of *C. mydas* to cardiovascular trematode eggs and found no relationship between the presence of ova and GTFP formation. The GTFP lesions did not represent a host response associated with papillary epidermal hyperplasia (Aguirre, 1994). In the present study, 7 of 12 (58%) ELISA-negative turtles exhibited mild to moderate fibropapilloma lesions (tumor score 2–3). Thus, this supports the lack of a direct relationship between GTFP and the exposure of green turtles to spirorchid trematodes.

It has been hypothesized that *C. mydas* hatchlings imprint to the chemical cues characteristic of their natal beach and return to the same beach for mating and nesting (Grassman, 1993). According to the chemical imprinting (Grassman, 1993) and natal homing hypotheses (Meylan et al., 1990), the same turtles repeat the same pattern of migration. Thus, the significant difference in the ELISA-Ab titer among green turtles from various discrete populations can be explained by the epizootiological differences in the habitat frequently visited by the same animals. Epizootiological studies on these parasites do not exist, and, so far, examination of snails for cercariae has yielded negative results (Greiner et al., 1980; Dailey et al., 1992). However, the present study indicates that the search for infected snails should be focused in the Keawanui Bay on the Island of Hawaii, and not in Ahu-O-Laka, Kaneohe Bay, Island of Oahu.

TABLE I. Size, weight, fibropapilloma severity score, and serological positivity for spirorchid blood flukes (*Learedius learedi, Hapalotrema dorsopora,* and *Carettacola hawaiiensis*) of the wild green turtles (*Chelonia mydas*) from Hawaii, 1991–1993.

Site (number of animals)	Range and mean straight carapace length (±SD) (cm)	Range and mean weight (±SD) (kg)	Range of tumor severity score*	Percentage of positive turtles†
Kaneohe Bay‡ Ahu-O-Laka (n = 41)	37.4–66.3 47.9 ± 6.8	7.7–35.8 15.4 ± 6.2	0–3	71
Mark Reef‡ (n = 2)	40.8–43.5 42.1 ± 1.9	9.5–11.0 10.3 ± 1.1	0	100
Reef 42‡ (n = 3)	45.9–68.3 56.5 ± 11.3	15.5–40.0 27.0 ± 12.3	3–4	100
Keawanui Bay§ (n = 10)	39.9–68.3 53.8 ± 8.7	9.0–38.5 21.2 ± 9.4	0	100
Haleiva and Kailua Bay‡ (n = 2)	69.4–85.7 77.5 ± 11.5	40.0–77.2 58.6 ± 26.4	3–4	100

* On a scale of 0–4, 4 being the most severe case.
† Determined by the indirect enzyme-linked immunosorbent assay utilizing surface glycocalyx crude antigen.
‡ Island of Oahu.
§ Island of Hawaii.

ELISA-detected heterogeneity of surface antigens related to the age of trematodes has been reported in human blood flukes (Ramalho-Pinto et al., 1978) and intestinal trematodes (Hockley and McLaren, 1973; Rivera-Marrero et al., 1988). In the present study, the preparation of the antigen from all recovered worms reduced the potential age-related heterogeneity of the antigen. Combining the surface glycocalyx antigen preparations from *L. learedi, H. dorsopora,* and *C. hawaiiensis,* allows for the detection of a broad spectrum of humoral responses. The combined antigen used in the assay can be potentially shared by helminths outside the target group. However, to date, no trematodes other than Spirorchinae (sensu Yamaguti, 1971) have been reported from the turtle blood system, and, as shown in the present study, the ELISA-detected humoral responses were specific to these flukes but not to the intestinal trematode *E. caproni.* The 80% prevalence of ELISA-positive *C. mydas* in the present study was significantly higher (G-test; $G = 4.91$, $P < 0.03$) than the 59% prevalence (16 of 27) of necropsy-positive animals from Australia (Glazebrook et al., 1989), and the 40% prevalence of turtles from Hawaii infected with *L. learedi* and *C. hawaiiensis* (30%) (Dailey et al., 1992) (G-test; $G = 13.1$, $P < 0.01$).

No significant differences in absorbance values were observed between the NC obtained by heating of the plasma in the presence of citric acid (Johnson et al., 1989), and the plasma that originated from the uninfected animals. Thus, in the absence of

plasma from a parasite-free turtle, the ELISA can utilize the NC generated from any positive turtle plasma.

The results of the present study demonstrate that the ELISA could be applied to diagnostic surveys of exposure of green turtles to spirorchid trematodes. The ELISA wells that gave absorbance of 0.12 or higher (the cutoff level was 0.14) at 405 nm wavelength can be clearly visually distinguished (particularly on a white background) from the negative wells. Thus, the need for an automated ELISA reader is eliminated, making this method suitable for field surveys. This test is expected to be a tool for evaluation of epidemiologic association of GTFP with spirorchid trematodes. The ELISA method can be used also for evaluation, assessment, or selection of turtles for pharmacological anti-blood fluke treatment when such becomes available.

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