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ELISA TEST FOR THE DETECTION OF ANTI-BLOOD FLUKE (*CARETTACOLA, HAPALOTREMA*, AND *LEAREDIUS*) ANTIBODIES IN JUVENILE GREEN TURTLES (*CHELONIA MYDAS*) WITH AND WITHOUT FIBROPAPILLOMAS IN THE HAWAIIAN ISLANDS

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This Administrative Report is issued as an informal document to ensure prompt dissemination of preliminary results, interim reports, and special studies. We recommend that it not be abstracted or cited.

PREFACE

This report was prepared in part as the result of contracts issued to Dr. A. Alonso Aguirre by the Southwest Fisheries Science Center Honolulu Laboratory's Marine Turtle Research Program. The report describes the development of an enzyme-linked immunosorbent assay (ELISA) utilizing the crude antigen of three species of trematodes for the detection of antibodies in the Hawaiian green turtle, *Chelonia mydas*, infected with these cardiovascular parasites. This ELISA test holds potential for the routine monitoring of green turtle aggregations in Hawaii to determine their exposure to trematodes. Epidemiological insights may result from such work as to when and where infections are acquired and their relationships, if any, to the often fatal tumor disease known as fibropapillomatosis.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

George H. Balazs
Zoologist
November 1994

EXECUTIVE SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was 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 in the plasma of the clinically infected turtle. Low absorbance values (< 0.074) of nonspecific background were observed. The gross lesions and histopathology were typical for cardiovascular spirorchidiasis in *C. mydas*. No significant relationship was found between the size of animals and the degree of GTFP severity. Forty-seven of 59 (80%) samples, originating from 5 sites, gave a positive reaction with the pooled blood fluke antigen; 6 of the 47 (13%) specimens gave significantly ($P < 0.001$) higher absorbance values, and 5 of them originated from the same location. All 12 (20%) ELISA-negative turtles originated from another site; and the absorbance values of the 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.

INTRODUCTION

The Hawaiian green turtle (*Chelonia mydas*) is a geographically isolated population protected under the U.S. Endangered Species Act and wildlife laws of the State of Hawaii. The population has shown a gradual increase since 1978 (Balazs, 1991). Cardiovascular spirorchidiasis caused by *Learedius learedi*, *Hapalotrema dorsopora*, and *Carettacola hawaiiensis*; however, is a trematodiasis that can affect the survival of the species and inhibit recovery (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 et al., 1994a). 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 (Fried, 1964; Smith, 1972) may not apply for *C. mydas* in Hawaii.

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 etiology (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 et al., 1994b), chronic stress (Aguirre, 1994), and a genetic predisposition to neoplasia (Balazs and Pooley, 1991). It has been shown (Aguirre and Spraker, 1994) that co-occurrence of spirorchidiasis and GTFP causes debilitation and fatality in juveniles of *C. mydas* in Hawaii.

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 habitats 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 has not been available. Additionally, 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.

MATERIALS AND METHODS

Turtle specimens for this study were obtained from different sources. Juvenile green turtles were captured alive and unharmed by hand while snorkeling during the month of September over 3 consecutive years (1991-93). Turtles were captured in three study sites in Kaneohe Bay, Island of Oahu: Ahu-O-Laka (n = 41), Mark Reef (n = 3), and Reef 42 (n = 2). In September of 1993, large-mesh tangle nets were used to catch 10 turtles at Keawanui Bay, Island of Hawaii. In addition, two turtles severely affected by GTFP and in poor condition were recovered through the NMFS Hawaiian Sea Turtle Stranding and Salvage Program in Haleiwa and Kailua Bay, Island of Oahu. A blood specimen was obtained from one clinically normal green turtle held in captivity for 5 years at the NMFS research facilities because of traumatic amputation of front flippers. Specimens from two turtles hatched in captivity at Sea Life Park, Island of Oahu, Hawaii, raised in an aquarium in Seattle, Washington, were tested as negative controls. These turtles were kept indoors under parasite-free conditions, and their diet consisted of human-grade squid.

Turtles collected in Kaneohe Bay were transported a short distance by boat to the research facilities provided by the Hawaii Institute of Marine Biology on Coconut Island, Kaneohe Bay. Upon arrival, turtles were measured, tagged, and weighed following techniques previously described (Balazs et al., 1987). Tumors in turtles with GTFP were evaluated based on the size, number, and location. Turtles were coded by degree of tumor severity on a scale of 1 to 4, 4 being the most severe case (Balazs, 1991).

Blood specimens (5-10 ml) were collected by venipuncture with a 10-ml syringe and a 20 ga needle. Samples were taken from the bilateral dorsal cervical sinuses (Owens and Ruiz, 1980), placed in heparinized Vacutainer tubes, centrifuged at 2,000 rpm for 10 minutes, and plasma stored in a Revco® ultrafreezer at -70°C until tested.

A moribund turtle severely affected with GTFP and with no possibility of surviving in the wild, was euthanized with a lethal intraperitoneal injection of Beuthanasia-D Special Solution (Schering-Plough Animal Health Corp., Kenilworth, New Jersey, USA). The turtle was exsanguinated, and its blood centrifuged at 10,000 rpm for 15'; plasma was placed in 10-20 ml aliquots and frozen at -70°C. A thorough necropsy was performed following the protocol described by Wolke and George (1981). Lungs, liver, heart and adjacent vessels, and mesenteric arteries were examined for endoparasites. Cardiovascular trematodes were placed in a petri dish containing PBS pH 7.4, counted, and identified to species under a dissecting stereomicroscope. Then, adult flukes were air-dried and stored in vials at -70°C in an ultrafreezer until tested. The identification of the specimens followed the description by Skrjabin (1964), Dawes (1968),

Yamaguti (1971), and Ernst and Ernst (1977). Standard techniques were followed for the histopathologic evaluation of specimens. Organ sections were fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned 6-μm thick and stained with hematoxylin and eosin (Aguirre and Spraker, 1994).

For the antigen preparation, the flukes were thawed, pooled, and each 6 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 protocol of Caufield et al. (1987). The concentration of the glycocalyx-containing fraction, determined by a Milton Roy Spectronic 1001 spectrophotometer (Milton Roy, Durham, North Carolina, USA) with absorbance at 280 nm wavelength, was 0.89 mg/ml. 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-reptilians/amphibians IgG conjugated to alkaline phosphatase to immunoglobulins in the plasma of *C. mydas*. Anti-reptiles/amphibians serum was generated in New Zealand white rabbits according to the protocol of Irby and Apperson (1988), and was a gift from C. S. Apperson. Purification of anti-reptilian/amphibian IgG from the serum by 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 sensitivity was achieved using Immulon 2 microtitration plates (Dynatech Laboratories Inc., Chantilly, Virginia). Eight wells on 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. The tested plasma was prepared by pooling of 0.25 ml of plasma from each of 60 samples. 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) and 100 μl of anti-reptilians/amphibians 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 h, 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 previously described (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 with antigen to determine nonspecific background values (NBV) of absorbance. Following

coating, the plate was postcoated by incubating each well for 3 h 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 plasma was a pool of the plasma obtained from the three green turtles from the NMFS research facilities and Sea Life Park. 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 and PC plasmas 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 thawing and freezing. Eight wells were filled with NC plasma, and 8 wells were filled with PC plasma. After 3 h incubation at 37°C the plates were washed 6 times with PBS/0.05% TW-20, 100 μl of anti-reptiles/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 indirect ELISA was performed 2 weeks later with the same 60 plasma samples, and following the same protocol.

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

Absorbance values 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 greater than the mean of absorbance ± 3 SD of NC plasma. 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 plasma was used in each plate. The mean adjustment from plate to plate was 1.86%, and the results did not differ when unadjusted absorbance values 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 from the means obtained from the same plates, and a G-test was used to compare the percentage of ELISA-positive animals among the groups. The degree of linear association between variables was compared using Pearson's correlation coefficient (*R*). Significance was considered to be $P < 0.05$. Other statistical treatment followed the procedures of Sokal and Rohlf (1981).

RESULTS

A total of 68 *H. dorsopora*, 24 *L. learedi* recovered from heart chambers and major vessels, and 7 *C. hawaiiensis* recovered from the hepatic vessels were identified in the green turtle used as positive control. The gross and histopathologic lesions were associated with spirorchid trematode infection and GTFP (Aguirre and Spraker, 1994). The turtle was severely emaciated and gross lesions included lobulated tumors of different size classes, serous atrophy of fat, and edema in subcutaneous tissues, pectoral and coracoid muscles; hydropericardium and pulmonary edema were also observed. A generalized thickening and hardening of major vessels, with partial or complete obliteration of blood flow and thrombi formation was observed. Histologically, a generalized lymphoplasmacytic endarteritis and thrombosis, and multifocal granulomas associated with trematode ova were observed throughout the parenchyma of several organs including salt glands, heart, lungs, gastrointestinal tract, spleen, and kidneys. The granulomas were composed of multinucleated giant cells and macrophages engulfing parasitic ova (Aguirre and Spraker, 1994).

In preliminary experiments, various dilutions of the crude surface glycocalyx membrane 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 specificity and sensitivity. Using the protocol for the indirect ELISA assay 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 caused the increase of 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, $x = 0.58 \pm 0.29$, (Fig. 1), and decreased significantly ($R = 0.73$, $P < 0.002$) with increasing 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 ELISA was 0.70 - 0.67; x = 0.68 ± 0.01), and 0.11 - 0.12; x = 0.11 ± 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 with 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, x = 646 + 0.02) than the rest of the positive samples (range: 0.550 - 0.311, x = 41 + 0.06), 5 of 6 of these samples originated from the same site (Keawanui Bay at the Island of Hawaii) (Table 1). The absorbance of 12 of 59 (20%) samples did not reach the cutoff level, and these 12 negative turtles originated from 1 site, Ahu-O-Laka at Kaneohe Bay on the Island of Oahu (Table 1). 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 1) 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.

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 plasma from 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 absorbance 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 specimens, it appears that they included 8 NC plasma and 3 samples determined to be negative by the previous indirect ELISA. The absorbance values of 31 samples were significantly (paired t-test; t = 155.75, P <0.001) higher than the absorbance of NC, and appear that all originated from positive animals.

DISCUSSION

Gross pathology found in the *C. mydas* from which the flukes were recovered confirmed the pathological findings associated with cardiovascular spirorchidiasis and GTFP (Aguirre and Spraker, 1994; Aguirre et al., 1994*a*). The site of infection of *C. hawaiiensis* in the present study confirmed the observation of Dailey et al. (1991) on exclusive occurrence of this species in hepatic vessels of marine turtles. During this study, the pathological changes associated with parasitic granulomas in the liver, spleen, lungs, and other tissues caused by *Learedius* sp., *Carettacola* sp., and *Hapalotrema* spp. have been previously

described in green turtles from Australia (Glazebrook et al., 1989). The histopathology and pathogenesis of parasitic granulomas associated with chronic vasculitis is described in detail for loggerhead turtles (*Caretta caretta*) from the Atlantic seaboard (Wolke et al., 1982). More recently, a detailed description of the gross and microscopic pathology of spirorchidiasis and its association to GTFP in Hawaiian green turtles was provided by Aguirre and Spraker (1994).

Parasitologic surveys in Hawaiian green turtles afflicted with GTFP (Dailey et al., 1992; 1993) demonstrated prevalences of 80-100% for *H. dorsopora*, 40% for *L. learedi*, and 30% for *C. hawaiiensis*. The distribution of infection with these trematodes apparently was not affected by size, sex, or location (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 prevalences of infection in a wild population of green turtles. 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 green turtle populations in Hawaii by determining their exposure to spirorchid trematodes. Monitoring the Ab titer over time in selected marked turtles may reveal epidemiologic insights on when and where trematode infections are acquired and their relationship to GTFP.

Jacobson et al. (1989) examined histological biopsies taken from cutaneous fibropapillomas in green turtle from Hawaii and from Indian River Lagoon System in Florida. They found trematode ova only in the biopsies taken from the Hawaiian population. As shown in the present study, the 7 of 12 ELISA-negative green turtles exhibited mild to moderate fibropapilloma lesions (tumor scores 2 and 3). In addition, the cellular response of green turtles to trematode egg exposure was recently evaluated (Aguirre, 1994). That study provided no evidence that spirorchid trematode eggs were the initial cause of fibropapilloma formation and that the lesions observed did not represent a host response associated with papillary epidermal hyperplasia. In the present study, 7 of 12 (58%) ELISA-negative turtles exhibited moderate to severe fibropapilloma lesions (tumor score 2 to 3). Thus, this supports the lack of a direct causal 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 theories (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 habitats frequented by these 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). 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.

ELISA-detected heterogeneity of surface antigen related to the age of trematodes has been reported in human schistosomes (Ramalho-Pinto et al., 1978), and in a variety of intestinal trematodes (Hockley and McLaren, 1973; Rivera-Marrero et al., 1988). In the present study, the preparation of the antigen from all recovered worms reduces the potential age-related heterogeneity of antigen. Combining the surface glycocalyx antigens from *L. learedi*, *H. dorsopora*, and *C. hawaiiensis*, allows for the detection of a broad spectrum of humoral responses. The 89% 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 negative control (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 can be applied to diagnostic surveys of exposure of green turtles to blood flukes. 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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Table 1.--Size, weight, fibropapilloma severity score, and serological positivity for spirorchid blood flukes (*Learedius learedi*, *Hapalotrema dorsopora*, and *Carettacola hawaiiensis*) immunoglobulins in plasma from wild green turtles (*Chelonia mydas*), Hawaii, 1991-93.

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

[a]On a scale of 0 to 4, 4 being the most severe case (Balazs, 1991)
[b]Determined by the indirect enzyme-linked immunosorbent assay (ELISA) utilizing surface glycocalyx crude antigen
[c]Island of Oahu
[d]Island of Hawaii

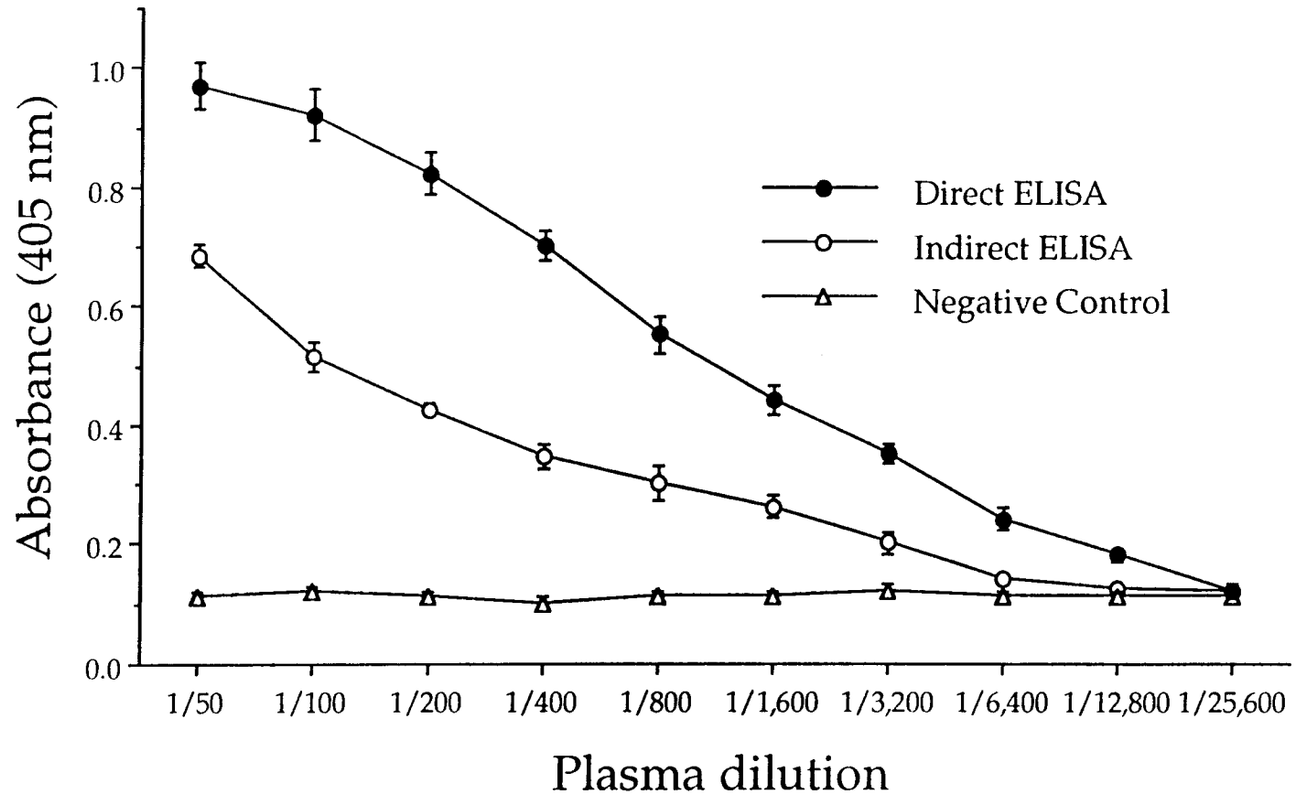

Figure 1.--Binding efficacy obtained in direct enzyme-linked immunosorbent assay (ELISA) for anti-reptilian/amphibian IgG labeled to alkaline phosphatase and immunoglobulins in the plasma of wild green turtles (*Chelonia mydas*), and detection of anti-blood flukes immunoglobulins by indirect ELISA in the plasma of a naturally infected turtle with fibropapillomas. Negative control plasma for indirect ELISA originated 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.