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Development and use of an indirect enzyme-linked immunosorbent assay for detection of iridovirus exposure in gopher tortoises (*Gopherus polyphemus*) and eastern box turtles (*Terrapene carolina carolina*)

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ABSTRACT

Iridoviruses, pathogens typically associated with fish and amphibians, have recently been shown to cause acute respiratory disease in chelonians including box turtles, red-eared sliders, gopher tortoises, and Burmese star tortoises. Case reports of natural infections in several chelonian species in the United States have been reported, however the prevalence remains unknown in susceptible populations of free-ranging chelonians. To determine the prevalence of iridovirus exposure in free-ranging gopher tortoises (*Gopherus polyphemus*) in the southeast United States, an indirect enzyme-linked immunosorbent assay (ELISA) was developed and used to evaluate plasma samples from wild gopher tortoises (*G. polyphemus*) from: Alabama ($n = 9$); Florida ($n = 658$); Georgia ($n = 225$); Louisiana ($n = 12$); Mississippi ($n = 28$); and unknown locations (68) collected between 2001 and 2006. Eight (1.2%) seropositive tortoises were identified from Florida and seven (3.1%) from Georgia for an overall prevalence of 1.5%. Additionally, a population of eastern box turtles was sampled from a private nature sanctuary in Pennsylvania that experienced an outbreak of iridovirus the previous year, which killed 16 turtles. Only 1 turtle out of 55 survivors tested positive (1.8%). Results suggest a low exposure rate in chelonians to this pathogen; however, it is suspected that this is an underestimate of the true prevalence. Since experimental transmission studies and past outbreaks have shown a high rate of mortality in infected turtles, turtles may die before they develop an antibody response. Further, the duration of the antibody response is unknown and may also cause an underestimate of the true prevalence.

1. Introduction

Iridoviruses of the genus *Ranavirus* are well known for causing mass mortality events of fish and amphibians with increasing reports of infection in reptiles. Prior to 2003, only two occurrences of chelonian infection with a *Ranavirus* were known in the United States; a free-ranging gopher tortoise (*Gopherus polyphemus*) in Florida (Westhouse et al., 1996) and a collection of captive box turtles (*Terrapene carolina carolina*) in North Carolina (DeVoe et al., 2004). Since then, several additional cases and outbreaks have been identified and described in both free-ranging native and captive exotic chelonian species including Eastern and Florida box turtles (*T. carolina carolina* and *T. carolina bauri*), gopher tortoises, and

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Burmese star tortoises (*Geochelone platynota*) (Allender et al., 2006; Johnson et al., 2008). Experimental transmission studies have confirmed that ranaviruses are in fact primary pathogens of chelonians (Johnson et al., 2007). The method of transmission has yet to be elucidated, but conspecific amphibians have been shown to be infected with identical or very closely related viruses, suggesting that amphibians may serve as a source of infection for chelonians (Johnson et al., 2008). The prevalence and incidence of infections in free-ranging chelonians remains unknown.

Indirect enzyme-linked immunosorbent assays (ELISA) have been used to detect exposure of various species of reptiles to specific pathogens (Schumacher et al., 1993; Origgi et al., 2001; Brown et al., 2001; Jacobson et al., 2005) and have been used to detect exposure of amphibians to iridovirus infections (Whittington et al., 1997; Gantress et al., 2003; Maniero et al., 2006). To determine iridovirus exposure in free-ranging gopher tortoises in the United States, an indirect ELISA was developed using a previously described mouse anti-desert tortoise IgY monoclonal antibody as the secondary antibody (Schumacher et al., 1993). We describe the assay and the results of a larger serological survey of free-ranging gopher tortoises from various sites in Alabama, Florida, Georgia, Louisiana and Mississippi. Additionally, 55 surviving eastern box turtles (*T. carolina carolina*) from a population in Pennsylvania that experienced an outbreak of illness that killed 16 turtles were sampled to determine whether any of the box turtles were exposed that might have survived infection.

2. Materials and methods

2.1. Positive and negative reference plasma and virus isolate

In July of 2003, three of five captive Burmese star tortoises (BST) became ill with clinical signs consisting of nasal discharge, conjunctivitis, frothing from the mouth, cervical subcutaneous edema, and oral plaques. One of the three tortoises died and histologic lesions were observed that were consistent with those seen in iridovirus infections in fish, amphibians and other reptiles, including the presence of intra-cytoplasmic inclusion bodies (Reddacliff and Whittington, 1996; Westhouse et al., 1996; Bollinger et al., 1999; Docherty et al., 2003). Molecular investigations demonstrated the presence of *Ranavirus* in various tissues (Johnson et al., 2008). This *Ranavirus* here termed BSTRV, was used as the antigen in the development of the ELISA. Surviving tortoises were treated with acyclovir (Glaxo Wellcome, Brentford, Middlesex, United Kingdom) and supportive care and all four tortoises survived. Plasma was collected at the time of the onset of clinical signs of disease during July and then again 5 months later in September 2003. Plasma from one of these tortoises collected in September was used as the positive control in development of the ELISA. Plasma from a Burmese star tortoise from a zoological collection with no known history of disease was collected to serve as negative reference plasma for the ELISA.

2.2. Antigen preparation

Terrapene heart-1 (TH-1) cells were grown to confluency in 225 cm^2 tissue flasks (Costar, Corning, NY). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60 mg/l; Sigma, St. Louis, MO), penicillin G (120,000 U/l), streptomycin (120,000 U/l) and amphotericin B (300 μg/l; Sigma). Cells were inoculated with a fourth passage of BSTRV and incubated at 28 °C in the presence of 5% CO_2. When cytopathic effects (CPE) consisting of cell rounding and detachment from the flask were observed in over 80% of cells, the cells and media were transferred to 15 ml tubes and centrifuged at 4500 × *g* for 30 min. Supernatant was then discarded and the cell pellets were resuspended in residual media, and frozen and thawed three times to release virus from the cells. Tubes were vortexed before and after each freeze cycle and following the final thaw. Subsequently, they were centrifuged again at 4500 × *g* for 30 min. Supernatant containing concentrated amounts of virus was then transferred to a 4 ml sterile cryotube. A Bradford protein assay was performed to determine the final protein concentration of the antigen (Bradford, 1976; BioRad, Hercules, CA). A previously described polymerase chain reaction (PCR) test was used to confirm the presence of the *Ranavirus* major capsid protein (MCP) gene (Marschang et al., 1999). Uninfected flasks were concurrently processed in the same manner to serve as control antigen to detect any background cross reactivity of plasma to cellular proteins.

2.3. ELISA procedure

A checkerboard optimization strategy was used to determine the optimum concentrations of both antigen and plasma to be used in the ELISA. Antigen concentrations were evaluated at dilutions of 1:100, 1:250, 1:500, and 1:1000. Plasma concentrations evaluated were 2-fold serial dilutions from 1:50 to 1:1600. The following procedure was found to be optimal utilizing the crude cell lysate antigen. Each well of a high protein binding 96 well microplate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated with 50 μl of infected or uninfected cell lysate diluted to 1:100 in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% sodium azide (PBS/Az). Plates were incubated overnight at 4 °C. Antigen was then aspirated off and wells were washed four times with ELISA wash buffer (PBS/Az with 0.05% Tween 20). This washing process was performed between each of the following steps. Wells were then blocked against non-specific binding with 300 μl of Superblock blocking buffer by Pierce (Rockford, IL) for 1 h at room temperature (RT). Each remaining step was incubated for 1 h at RT. Plasma samples diluted 1:100 in blocking buffer were added at 50 μl volumes to wells in triplicate. One well was coated with uninfected cell lysate, while the other two wells were coated with infected cell lysate. Next, a biotin-conjugated monoclonal antibody produced against the desert tortoise IgY light chain (Schumacher et al., 1993) was diluted to a final concentration of 0.5 μg/ml in PBS/Az and added to

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each well in 50 μl volumes. Alkaline phosphatase-conjugated streptavidin (Zymed Laboratories, Inc., San Francisco, CA) was applied to each well at 50 μl of a 1:5000 dilution in PBS/Az. Next, the ELISA was developed with 100 μl per well of a 1.0 mg/ml *p*-nitrophenyl phosphate prepared in 0.01 M sodium bicarbonate buffer containing 2 mM $MgCl_2$ and plates were stored in the dark. The absorbance of each well was read at A_{405} using a StatFax 3200 microplate reader (Awareness Technology, Palm City, FL) at 30 min.

Each plasma sample was read in triplicate. Plasma was placed on one well originally coated with uninfected cell lysate and on two wells coated with infected cell lysate. The average absorbance reading of the two wells coated with infected cell lysate was calculated and the positive/negative (P/N) ratio value of each sample was determined by dividing the mean absorbance of the duplicate average by the absorbance reading of the well from the uninfected cell lysate. This subtracts out any background noise, or non-specific binding that may have occurred as a result of using the *Terrapene* heart cell line. The cut-off value for a positive test result was determined by adding three times the standard deviation to the mean P/N ratio obtained (Crowther, 2001).

2.4. Reproducibility

Intra-assay and inter-assay reproducibility were determined by performing two precision runs. The positive and negative reference plasma samples, used to optimize the test, were used in each assay. Intra-assay reproducibility was determined by running the positive and negative sample multiple times on the same plate. Each sample was run multiple times on one well of uninfected cell lysate and two wells of infected cell lysate. This resulted in 64 readings for each sample on the infected cell lysate and 32 on the uninfected cell lysate. Inter-assay reproducibility was determined by using the values of the reference plasma results used as controls in assaying the wild gopher tortoise samples from multiple dates and multiple plates. The mean A_{405}, the standard deviation (SD) and the coefficient of variation (CV) for the intra- and inter-assay reproducibility were calculated using the optimized ELISA conditions.

2.5. Protein expression and immunoblotting

The positive control plasma was tested for its ability to detect viral proteins in a western blot using infected and uninfected TH-1 cell lysates as antigen. Four 4-fold dilutions of infected and uninfected cellular lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions along with broad range molecular weight markers. The separated proteins were transferred onto 0.2 μm nitrocellulose membranes (Biorad, Hercules, CA) by standard methods (Harlow and Lane, 1988). Membranes were then rinsed in water for 5 min in preparation for coomassie blue staining or in Tris-buffered saline containing 0.5% Tween 20 (TTBS, pH 7.5) for 20 min prior to immunoblotting. Membranes were then stained in coomassie blue stain for 90 min, followed by water for 30 min to destain to view differences in protein profiles between infected and uninfected cell lysates. After rinsing in TTBS, membranes for immunoblotting were blocked with Superblock blocking buffer in phosphate buffered saline (Pierce) for 1 h. Blocking buffer was then removed and plasma samples diluted 1:2000 in blocking buffer were added to the membranes. After 1 h, the membranes were washed with TTBS for 30 min and the monoclonal antibody, diluted 1:10,000 in blocking buffer, was added and incubated for 1 h. Again the membranes were washed and AP streptavidin, diluted 1:5,000 in phosphate buffered saline was added and allowed to incubate for 1 h. Membranes were washed as previously described, and the membranes were developed in substrate buffer (0.1 M Tris–HCl, 1 mM $MgCl_2$) containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (Biorad). The reaction was stopped by removing the NBT–BCIP solution and adding deionized water. Membranes were allowed to air dry.

2.6. Free-ranging gopher tortoise samples

Plasma samples from 1000 wild gopher tortoises (*G. polyphemus*) from Alabama ($n = 9$), Florida ($n = 658$), Georgia ($n = 225$), Louisiana ($n = 12$), Mississippi ($n = 28$) and unknown locations (68) were obtained from recently submitted samples to the Mycoplasma Research Laboratory at the University of Florida between 2001 and 2006. County, state and date of sample collection was recorded for each plasma sample when it was available.

2.7. Box turtle samples

During the summer and fall of 2003, a population of 71 repatriated eastern box turtles (*T. carolina carolina*) in a private nature sanctuary in Pennsylvania, regularly tracked by radiotelemetry for over 4 years, experienced an unprecedented occurrence of sudden deaths in 16 turtles. The outbreak was investigated and iridovirus infections were confirmed in three of the turtles as previously described (Johnson et al., 2008). It is suspected to be the cause of death in the other 13 turtles, however further diagnostic work-up was not performed in these turtles. In 2004, the surviving 55 turtles were sampled. Plasma samples were stored on ice and transported to the University of Florida for testing.

3. Results

3.1. ELISA parameters

Results of the checkerboard optimization strategy determined that a 1:100 dilution of both the antigen and plasma samples gave the largest difference of P/N ratios between the positive and negative reference samples (Fig. 1). The frequency distribution of the P/N ratio is shown in 0.1 increments from 0.5 to 4.1 (Fig. 2). The mean P/N ratio of samples (1.078), plus three times the standard deviation (0.379) was used to set the positive cut-off value at 2.2. P/N ratios from each sample were plotted

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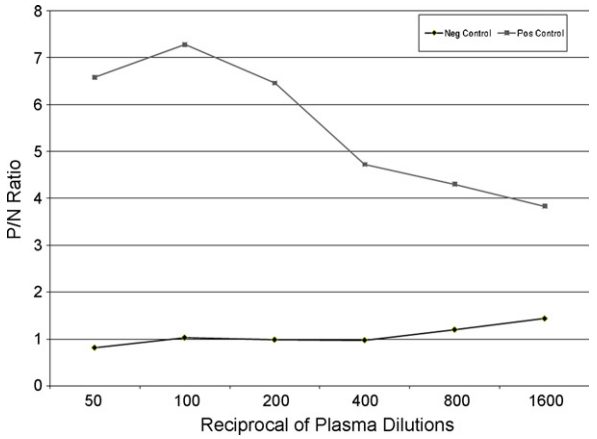

Fig. 1. Optimization of the ELISA with antigen coated at 1:100 dilution, comparing the positive to negative (P/N) ratio of 2-fold serial plasma dilutions of the positive control turtle (Burmese star tortoise with clinical signs of illness) versus a negative control (Burmese star tortoise with no history of illness). Plasma diluted at 1:100 showed the greatest difference between the positive and negative control.

on a graph with the sample numbers on the *x*-axis in increasing order and the P/N ratio values on the *y*-axis (Fig. 3).

3.2. Reproducibility

The mean A_{405}, SD and CV values for the intra- and inter-assay precision runs are shown in Table 1. Ideally, CV values should be less than 15% (Crowther, 2001). Three of the CV values in the inter-assay precision runs were >15% indicating that some variability still existed in this assay when performed at different time periods. Intra-assay coefficients of variance were less than 15%.

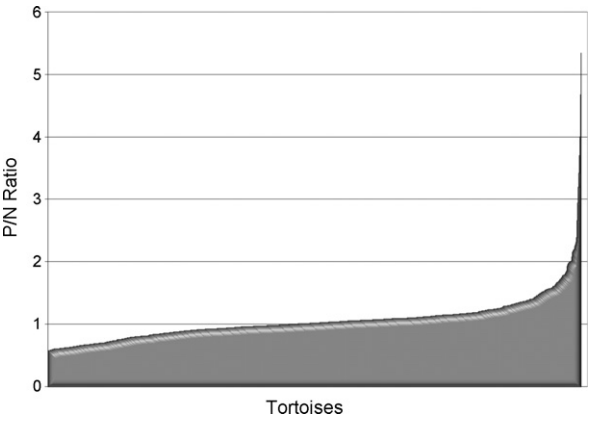

Fig. 3. Individual P/N ratio values for 1000 free-ranging gopher tortoises (*Gopherus polyphemus*) in increasing value.

3.3. Protein expression and immunoblotting

Coomassie blue staining of proteins from infected cell lysates and uninfected cell lysates showed a different pattern of protein expression (Fig. 4). Immunoblotting of infected and uninfected cell lysate showed a marked increase in binding to proteins in the infected cell lysate and very weak binding to proteins in the uninfected cell lysate (Fig. 5). Strong signals were seen on virus-infected cells at approximately 125 kDa, and 78 kDa with weaker signals seen at 70, 65, and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the uninfected cells.

3.4. Free-ranging gopher tortoise samples

Of 1000 gopher tortoise plasma samples assayed, 8 (1.2%) tortoises from Florida and 7 (3.1%) from Georgia

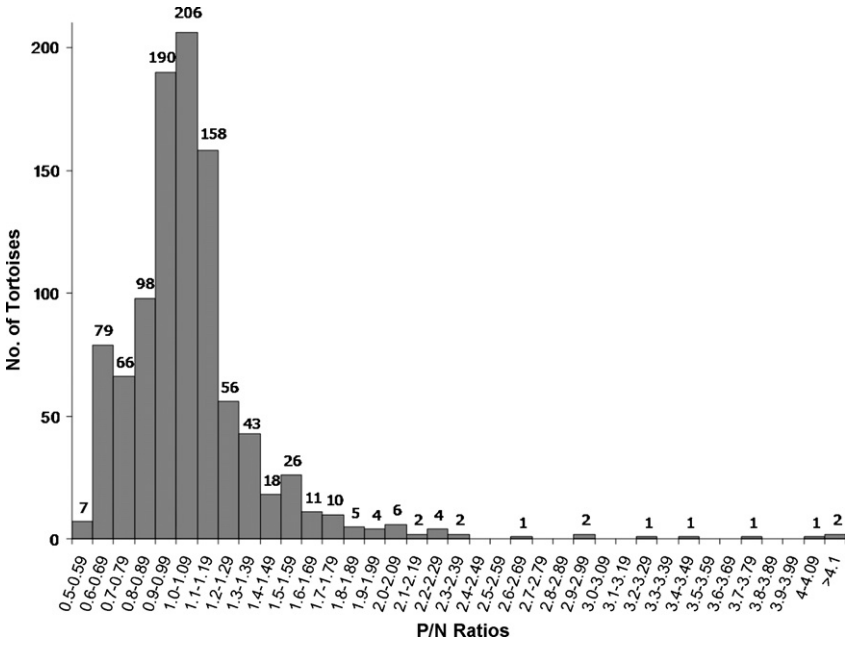

Fig. 2. Frequency distribution of P/N ratios from an indirect ELISA performed on 1000 free-ranging gopher tortoise (*Gopherus polyphemus*) plasma samples.

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Table 1
Reproducibility of the ELISA.

		Positive sample				Negative sample			
		n	Mean A_{405}	SD	CV	*n*	Mean A_{405}	SD	CV
Intra-assay	ICL	64	0.363	0.022	6.06	64	0.107	0.011	10.28
	UCL	32	0.094	0.009	9.57	32	0.112	0.009	8.04
Inter-assay	ICL	26	0.366	0.079	21.58	26	0.130	0.023	17.69
	UCL	13	0.095	0.011	11.58	13	0.111	0.017	15.31

SD, standard deviation of the mean A_{405}; CV, coefficient of variance expressed as a percent. ICL, values from samples run on wells coated with infected cell lysate; UCL, values from samples run on wells coated with uninfected cell lysate.

were seropositive, for a total of 1.5% of tortoises seropositive with a P/N ratio $\geq$2.2 (Table 2). All tortoises from Alabama, Louisiana and Mississippi were negative, as were the ones from unknown locations. Seropositive tortoises in Florida were located in five counties including Lake, St. Lucie, Broward, Palm Beach and Martin (Table 3, Fig. 6). Four of these are clustered together in the south including Palm Beach, Broward, Martin, and St. Lucie. The remaining seven seropositive tortoises were located in Baker, GA, a county in the southwest corner of the state (Fig. 6).

Date of sample collection was recorded in 680 samples. Two-thirds of the positive samples were collected in 2005 (Table 4), of which seven were from tortoises in Baker County, GA. One positive sample each was collected in 2003 and 2004, two positive samples in 2006 and no samples tested positive from those collected in 2001 and 2002.

3.5. Box turtle samples

Of 55 samples, only one box turtle tested positive with a P/N ratio of 7.2 for a prevalence of 1.8%. All other samples had a P/N ratio less than 1.7. No clinical signs of illness were observed in the turtle that tested positive, and the turtle, which was being regularly tracked by radiotelemetry had no known history of illness.

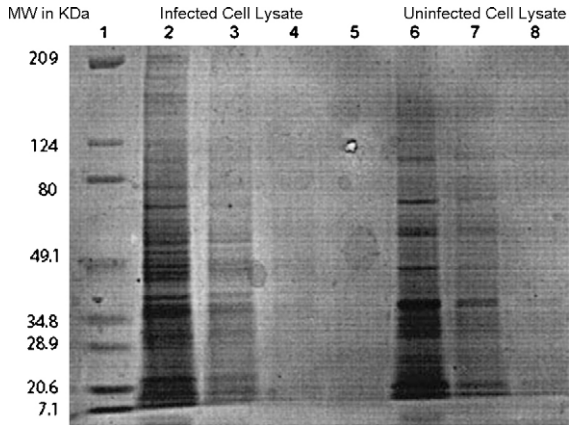

Fig. 4. Coomassie blue staining of a SDS-PAGE gel separating proteins of iridovirus-infected and uninfected *Terrapene* heart cell lysates. Lane 1 is a broad range, pre-stained molecular weight marker with weight in kDa marked next to the lane. Lanes 2–5 are 2-fold serial dilutions of iridovirus-infected cell lysate. Lanes 6–8 are 4-fold serial dilutions of uninfected cell lysate.

4. Discussion

This study documents the development of an assay to detect anti-iridovirus antibodies and the first report of anti-iridovirus antibodies in free-ranging and captive chelonians. The ELISA detected anti-iridovirus antibodies 2 months after 2 Burmese star tortoises recovered from an upper respiratory illness with oral plaques, which had been previously housed with a tortoise that died with confirmed iridovirus infection (Johnson et al., 2008). Plasma from a Burmese star tortoise from another facility with no known history of illness was selected as a

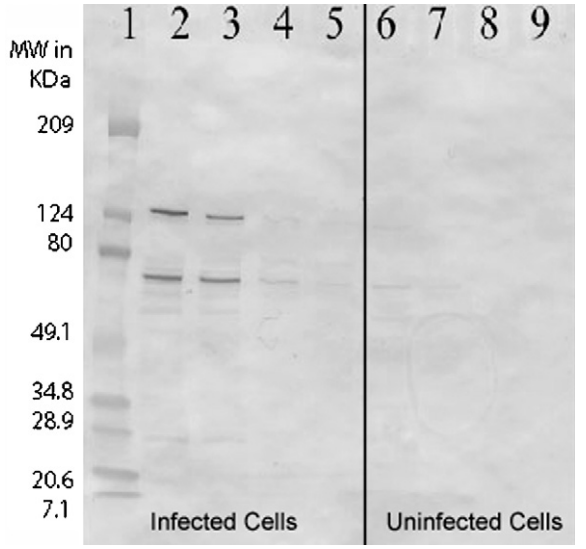

Fig. 5. Western immunoblot. Lanes coated with 4-fold serial dilutions of iridovirus-infected cell lysate in lanes 2–5 and uninfected cell lysate in wells 6–9. Plasma from the positive control was used as the primary antibody for detection of iridovirus specific antibody binding. Strong signal was seen on virus-infected cells at approximately 125 kDa, and 78 kDa with weaker signals seen at 70, 65, and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the uninfected cells.

Table 2
ELISA results of plasma samples from free-ranging gopher tortoises (*Gopherus polyphemus*) by state.

State	Total no. tested	Total no. positive	Percent
Alabama	9	0	0
Florida	658	8	1.2
Georgia	225	7	3.1
Louisiana	12	0	0
Mississippi	28	0	0

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Table 3
Anti-iridovirus ELISA results of 1000 free-ranging gopher tortoise (*Gopherus polyphemus*) plasma samples by county.

State	County	No. tested	No. positive	Percent
Alabama	Baldwin	2	0	0
	Mobile	7	0	0
Florida	Alachua	12	0	0
	Brevard	27	0	0
	Broward	10	1	10
	Citrus	51	0	0
	Clay	1	0	0
	Collier	2	0	0
	Columbia	1	0	0
	Hernando	24	0	0
	Hillsborough	16	0	0
	Indian River	2	0	0
	Lake	99	3	3
	Lee	18	0	0
	Leon	17	0	0
	Madison	1	0	0
	Manatee	6	0	0
	Marion	22	0	0
	Martin	25	1	4
	Miami-Dade	8	0	0
	Nassau	47	0	0
	Orange	40	0	0
	Osceola	11	0	0
	Palm Beach	58	2	3
	Pasco	26	0	0
	Pinellas	2	0	0
	Polk	6	0	0
	Sarasota	1	0	0
	Seminole	54	0	0
	St. Johns	7	0	0
	St. Lucie	35	1	3
	Taylor	1	0	0
	Volusia	26	0	0
	Walton	2	0	0
Georgia	Baker	113	7	6
	Liberty	74	0	0
	Tattnall	38	0	0
Louisiana	Washington Parish	12	0	0
Mississippi	Greene	7	0	0
	Harrison	16	0	0
	Perry	5	0	0
	Unknown	68	0	0
Total		1000	15	1.5

seronegative control and no antibodies to iridovirus were detected in this tortoise. Additionally, using a western blot, we were able to show that plasma from the positive control turtle sample bound to proteins in the infected cells in the immunoblot, but not in the uninfected cells indicating that the plasma was reacting with viral proteins and not cellular proteins from the lysate that was used to coat the plates. For these reasons, we consider this a valid test for use in detecting anti-iridovirus antibodies in chelonians, however further validation of this assay against another diagnostic assay is warranted to establish the sensitivity and specificity of the assay. Additionally, inter-assay precision runs indicated that some variability still exists when the assay is run at different time periods. The values for samples run on infected cell lysate were more variable than those run on uninfected cell lysate. This suggests that the technique for preparing antigen may vary, either due to differences because of the operator, or in the assay and/or reagents.

Humoral immune responses are characterized by an early response as indicated by an acute increase in plasma IgM levels, followed by an increase in IgG levels in mammals, or IgY levels in reptiles. Early studies using the anti-desert tortoise IgY light chain monoclonal antibody found that the antibody would detect the light chains of both IgM and IgY (Schumacher et al., 1993). This suggests that this ELISA can detect both recent infections with iridovirus as well as infections that may have occurred awhile before the plasma was collected. In the same study by Schumacher et al., seroconversion following inoculation with *Mycoplasma agassizii* occurred between 1 and 3 months (Schumacher et al., 1993), suggesting that in animals with positive ELISA results, exposure to iridovirus occurred at least 1 month prior to the date the sample was collected.

Prevalence among free-ranging gopher tortoises utilizing this assay was found to be low; only 1.5% of 1000 samples were positive. This could be the true prevalence rate, although we suspect that this is an underestimation. Prevalence is a function of the incidence of disease multiplied by the average duration of the illness. While incidence would be difficult to determine in a natural setting, duration of illness can be extrapolated from experimental studies. If chelonians die quickly as a result of infection, they will not have time to develop an immune response to the pathogen, and will not survive to be surveyed. As previously mentioned, experimental transmission studies have shown a high rate of mortality (75%) in turtles intramuscularly inoculated with 1 ml of 10^5 $TCID_{50}$/ml of an iridovirus-infected cell lysate (Johnson et al., 2007). Turtles that succumbed to the virus died within 30 days of exposure. If naturally exposed cases experience similar mortality rates and duration from exposure to death, a cross-sectional study evaluating the prevalence of exposure will miss many tortoises that were exposed, because most tortoises will die. This was demonstrated in a natural setting in Pennsylvania. Seventy-one radiotelemetered eastern box turtles (*T. carolina carolina)* were being followed by researchers since 1999 in a private nature sanctuary. Turtles had been translocated from another preserve in 1999 and experienced no disease outbreaks until 2003. In the summer and fall of 2003, 16 of these turtles died suddenly, with what was later identified to be iridovirus infections in 3 of the turtles that were tested (Johnson et al., 2008). The following spring, plasma from the surviving 55 turtles was evaluated by ELISA for the presence of anti-iridovirus antibodies. Only one turtle was positive on ELISA. If we calculated this value as the true prevalence, we would estimate that about 1.8% (1/55) of turtles were exposed, when we know that 24% (17/71) of the population was actually exposed, but 22% (16/71) died and so were not available to be included in the estimate. This severely underestimates the prevalence of disease. However, we cannot extrapolate these differences to other populations, as this was a repatriated population, and thus, subject to stressors that might not be found in naturally occurring populations, making them more susceptible to disease. Additionally, we are unsure how long anti-iridovirus antibodies remain at detectable levels in chelonians, or if all animals infected with the virus will mount an

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Fig. 6. Map showing counties in Alabama, Florida, Georgia, Louisiana and Mississippi. Counties highlighted in light gray represent the counties were tortoise samples originated. Counties in dark gray had tortoises tested that were seropositive for exposure to an iridovirus.

antibody response. If antibody levels decline prior to testing, or they never develop, then animal test results may be falsely negative, also creating an underestimate of prevalence.

Results of this serosurvey showed that counties in the southeastern region of Florida were more likely to have seropositive tortoises. Interestingly, four of these counties had adjoining borders (St. Lucie, Martin, Palm Beach and Broward), suggesting that either tortoises in this area are exposed to iridoviruses at a higher rate, or tortoises in this area are more resistant to disease, due to genetic or environmental factors. Environmental persistence of iridoviruses is poorly understood, however it appears moisture and temperature may play important roles. Studies with insect iridoviruses found that moisture was associated with increased persistence, while dessication inactivated viruses (Williams et al., 2005). Temperature has also been shown to play an important role in the infectivity of salamanders to iridoviruses, where salamanders exposed at lower temperatures are more susceptible to the virus than those infected at higher temperatures (Rojas et al., 2005). It is possible that tortoises infected with the virus in a warmer climate may be more resistant to infection, allowing them to develop an immune response and survive. However, this would not account for the higher prevalence rate of exposure of tortoises tested in Baker County, GA. Direct transmission may play a role in infection rates. In amphibians, activities involving close contact have been shown to be effective means of transmitting *Ranavirus* (Brunner et al., 2007). Box turtles have been shown to have overlapping home ranges, and mating occurs among turtles with overlapping ranges (Stickel, 1950, 1978, 1989). Interactions between males are similar to courtship behaviors between males and females (Stickel, 1989) and box turtles are often found near other box turtles (Dodd, 2001). Gopher tortoises of opposite sex often have overlapping home ranges and can commonly occupy the same burrow (Diemer, 1992). Therefore, it is possible that differences in population densities or overlapping home ranges may play a role in increased levels of exposure.

Table 4
Total number of samples collected each year and those that tested positive for anti-iridovirus antibodies with the county and state.

Year	No. tested	No. positive	County, state
2001	4	0	
2002	84	0	
2003	189	1	Palm Beach, FL
2004	124	1	Lake, FL
		7	Baker, GA
2005	178	1	Lake, FL
		1	Martin, FL
		1	St. Lucie, FL
2006	101	1	Broward, FL
		1	Palm Beach, FL
Unknown	320	1	Lake, FL

Earlier studies have shown that amphibians carry identical or very closely related iridoviruses to those found in the chelonians, suggesting they might be a source of infections (Johnson et al., 2008). Green et al. (2002) found that increased precipitation and population densities were directly associated with increased die-offs of amphibians due to iridoviruses. Other conditions that may cause stress and compromise the immune system have also been shown to increase susceptibility to iridoviruses. Forson and Storfer (2006) found that atrazine and sodium nitrate resulted in decreased peripheral leukocyte levels and increased susceptibility of salamanders to a *Ranavirus*. Gray et al. (2007) found that in some species of amphibians, stress resulting from sharing wetlands with

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cattle resulted in increased susceptibility of tadpoles to iridovirus infection. Similar conditions that cause stress to chelonians might also result in increased levels of susceptibility in chelonians. Additionally, it has been shown that sublethally infected amphibians can cause sporadic, recurrent disease outbreaks in amphibian populations (Brunner et al., 2004). Experimentally and naturally infected tiger salamander larvae and metamorphs were able to maintain sublethal, transmissible infections for over 5 months. Apparently healthy infected dispersing metamorphs were returning to water bodies to breed and it was speculated that these individuals were likely serving as a reservoir host for infecting newly hatched larvae, creating recurrent outbreaks of disease. It is unknown whether chelonians are capable of sustaining a subclinical persistent infection. This assay has conservation value since it can be used to identify asymptomatic carriers in wild and captive populations.

5. Conclusion

This study reports the development and use of an indirect ELISA for detection of anti-iridovirus antibodies in chelonians. Overall seroprevalence of exposure of free-ranging gopher tortoises to iridoviruses appear to be low, however, this is expected to be an underestimate of the true prevalence. Further studies are needed to characterize the impact of this viral disease on gopher tortoises and other chelonian species.

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