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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