EFFECTS OF RANAVIRUS INFECTION OF RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) ON PLASMA PROTEINS


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EFFECTS OF RANAVIRUS INFECTION OF RED-EARED SLIDERS (TRACHEMYS SCRIPTA ELEGANS) ON PLASMA PROTEINS


Abstract: Ranavirus is an emerging disease that infects fish, amphibians, and reptiles. Ranavirus induces an inflammatory response leading to death in many susceptible species. Red-eared sliders (RES; Trachemys scripta elegans) are vulnerable to ranavirus infection and are economically significant chelonians kept in the pet trade and utilized in research. Early identification of RES with inflammatory diseases would allow for isolation of affected individuals and subsequent disease investigation, including molecular testing for ranavirus. Validation of an inexpensive, clinically relevant, and reproducible diagnostic test that detects inflammation in turtles is needed. Although commonly used, plasma protein electrophoresis to detect an inflammatory acute-phase protein response has not been evaluated in a controlled environment in turtles with experimentally induced inflammatory disease. The objective of this study was to measure plasma protein fractions by electrophoresis to determine if an acute-phase protein response occurs in RES during infection with a frog virus 3-like ranavirus (FV3-like virus) isolated from a chelonian. A Bradford assay and agarose gel electrophoresis (AGE) were performed using plasma collected during a study of the effect of temperature on the pathogenesis of ranavirus in RES. In RES at the time of viremia, total albumin (ALB mg/ml) and albumin to globulin ratio were significantly lower and β-globulin percentage was significantly higher in RES exposed to ranavirus (n = 4) as compared to matched, uninfected RES (n = 8). In the last sample collected prior to death, total protein (TP mg/ml), ALB mg/ml, α-globulin percentage, and total α-globulin (α mg/ml) were significantly lower in RES exposed to ranavirus (n = 4) than control individuals (n = 8). In summary, FV3-like virus induces a decrease in plasma albumin concentration at the onset of viremia and decreases in TP mg/ml, ALB mg/ml, and α mg/ml concentrations prior to death in RES as measured by AGE.

Key words: Acute-phase protein, albumin, electrophoresis, globulin, ranavirus, turtle.

INTRODUCTION

The acute-phase response is a biological reaction to trauma or infection that helps to control injury induced by invading pathogens, mediate tissue damage, and promote a rapid return to homeostasis. During this response, hepatic and extrahepatic synthesis of a heterogeneous group of acute-phase proteins is altered. Acute-phase protein concentrations can be a crucial part of a diagnostic evaluation in species where more classic signs of inflammation are not present. The diagnosis of inflammation typically is not problematic in equine, canine, and feline species because an inflammatory leukogram often can be detected by performing a complete blood count (CBC). Acute-phase protein concentrations are used in these species to confirm that inflammation is occurring, guide treatment, and provide useful prognostic information. In cattle, however, the peripheral blood leukocyte response to inflammation is blunted, often not showing significant changes until severe disease is present. As a result, evaluation of acute-phase proteins to detect inflammatory disease in bovine medicine is commonplace. Peripheral blood cell counts are routinely performed in reptiles; however, leukocytosis is not reliably observed during markedly inflammatory disease processes. In the turtles used in this study, there were no significant differences between CBC results of ranavirus-infected turtles as compared to uninfected turtles despite the histologic evidence of marked inflammation (splenitis, vasculitis, and pneumonia) in the infected turtles. The inability to detect inflammation using CBC results has led to utilization of acute-phase proteins for routine monitoring of inflammatory disease in several reptilian species. However, scientific data to support this practice are limited. One controlled study evaluated hepatic transcription of acute-phase proteins in Chinese soft-shelled turtles (Trionyx sinensis) experimentally exposed to the freshwater bacteria Aeromonas hydrophila. These animals had hepatic transcript changes consistent with an acute-phase response during inflammation, including an increase in fibrinogen transcripts and a decrease in albumin transcripts. Hepatic biopsy to measure
acute-phase protein transcripts using real-time quantitative polymerase chain reaction (PCR) is invasive and likely would be inappropriate as a diagnostic tool in conservation and patient management efforts. Instead, there has been a call for a well-controlled investigation into the validity of using plasma protein electrophoresis for detecting an inflammatory acute-phase protein response in reptiles.\textsuperscript{15,39}

In mammals, protein electrophoresis is commonly performed using serum, rather than plasma. It is known that the correlation between canine plasma and serum protein electrophoresis is poor.\textsuperscript{8} Most studies using electrophoresis evaluate plasma proteins in reptiles, as plasma is easier to collect because of patient size and limited sample volume. Therefore, development of plasma protein reference intervals in reptiles, distinct from serum protein reference intervals, is needed.

Comparison of different plasma electrophoretic methods has shown that, for healthy red-eared sliders (RES; Trachemys scripta elegans) and green iguana (Iguana iguana), agarose gel electrophoresis (AGE) is preferred.\textsuperscript{15} Therefore, this study uses AGE to compare plasma acute-phase protein concentrations in RES ($n = 4$) at several time points during a controlled experimental infection with a frog virus 3–like ranavirus (FV3-like virus) that was isolated from a chelonian. The results from infected RES were compared to those from matched uninfected RES ($n = 8$). Additional clinical and histologic data from this experiment have been published and are available for review.\textsuperscript{1}

FV3-like virus induces a marked fibrinoid necrosis, heterophilic splenitis, vasculitis, and mild to moderate heterophilic pneumonia in experimentally infected RES.\textsuperscript{4} Because the histologic lesions in FV3-like virus–infected RES are inflammatory, it is reasonable to propose that ranavirus would induce an acute-phase protein response. Ranavirus is a clinically important member of the Iridoviridae family that has been associated with significant morbidity and mortality in wild amphibian and chelonian populations.\textsuperscript{8,16,28} It has recently been placed on the World Organization for Animal Health reportable disease list for amphibians, and increased work in understanding its pathogenesis and epidemiology have been undertaken.\textsuperscript{22,26,27,42} Early detection of inflammation would help ongoing conservation efforts by prompting researchers to isolate and test affected animals for ranavirus infection.

The specific biological hypothesis evaluated in this study is that ranavirus will induce an acute-phase protein response in RES, which can be measured by AGE as a decreased albumin percentage and concentration and increased globulin fraction percentages and concentrations.

**MATERIALS AND METHODS**

All animal activities were approved by the University of Illinois Animal Use and Care Committee (Protocol 11050). Heparinized plasma from 12 captive-raised RES was collected during an investigation into the effect of temperature on the pathogenesis of ranavirus after intramuscular exposure to $5 \times 10^6$ of a 50\% tissue culture infective dose (TCID$_{50}$) FV3-like virus, as described elsewhere.\textsuperscript{1} Control turtles were similarly inoculated with the same volume of sterile saline. In brief, 12 adult female captive-raised RES were acclimated to 22\°C for 1 wk. Four individuals were included in the exposed group with two individuals in the control group matched to each exposed individual (a total of eight control individuals). Heparinized plasma was collected from all individuals 7 and 4 days prior to FV3-like virus or saline inoculation. Following exposure, all individuals were phlebotomized twice weekly until the members of the exposed group were euthanized because of severity of clinical signs (nasal and ocular discharge, oral plaques, lethargy, leg swelling, and dermal ulcerations). Two matched individuals from the control group were euthanized concurrently with each exposed individual. Samples were stored at $-20^\circ\text{C}$ for 1 yr prior to testing.

Because the initial experimental exposure of the turtles was completed before conception of this study, alteration in sample size was not possible without complete recapitulation of the previous study. Although the sample size was small, utilizing the plasma from the previously completed study was deemed likely sufficiently sized to detect changes in the key proteins of interest and provide data that can be used to inform future experimental efforts.

Heparinized plasma from a quarter horse foal (Equus ferus caballus) with septic arthritis and pooled heparinized plasma collected during a surveillance effort of apparently healthy wild eastern ornate box turtles (Terrapene carolina carolina) also were used for procedural control purposes. These control plasma samples were stored at $-80^\circ\text{C}$ for 2 mo (horse) or $-20^\circ\text{C}$ up to 1 yr (box turtle) prior to testing.

AGE was used as described elsewhere.\textsuperscript{25} Briefly, the plasma was thawed and recentrifuged to produce platelet-poor plasma, which was diluted.
A Bradford assay (to determine total plasma protein concentration) and plasma electrophoresis were performed in duplicate. Resulting electrophoresis gels were stained with Coomassie blue and the relative densities of separated protein fractions were determined using a Kodak 440CF (Eastman Kodak Co., Rochester, New York 14650, USA) and Carestream Molecular Imaging Software (Carestream Health, Rochester, New York 14608, USA) with manual assignment of protein fractions similar to previously reported work.15 These data were imported into Excel software (Microsoft Corporation, Redmond, Washington 98052, USA) and protein fraction concentrations in the plasma samples were calculated.

Four time periods were defined for statistical evaluation: 1) pre-exposure (Pre-exp): both samples collected prior to the date of exposure were measured and averaged; 2) postexposure (Post-exp): a single plasma collection 11 days after the date of exposure, before clinical signs of disease in the exposed group; 3) Viremia: the first sample with quantitative PCR–verified viremia in the exposed turtles and the same time in the matched control turtles (7–21 days prior to death); 4) Last: the last sample collected before death (1–4 days prior to death). Insufficient plasma was available from one control turtle to test at Post-exp.

Evaluation of Pre-exp data for descriptive purposes utilized a Shapiro-Wilks test for normality followed by calculation of mean and composition of a histogram as recommended by the American Society for Veterinary Clinical Pathology for 10 < n < 20.12 Given that the exposed group had an n = 4, comparison of exposed to nonexposed groups was accomplished using a one-tailed Mann-Whitney U test to detect differences at a level of P-value < 0.05. As all tests could not be run at the same time, an aliquot of equine and an aliquot of pooled box turtle heparinized plasma were included as a procedural control with each run, and an intrarun reliability coefficient was calculated as recommended by Nunnally.34 Fleiss’s recommendations that a reliability coefficient >0.75 is excellent, 0.40–0.70 is fair to good, and <0.40 is less than desirable were used.9 Statistical evaluation was performed using GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, California 92037, USA), GraphPad StatMate 2 (GraphPad Software, Inc.), and Excel.

RESULTS

Figure 1 illustrates representative electrophoretograms of plasma collected from a turtle prior to ranavirus infection (Fig. 1A) and at the time that ranavirus was detected by quantitative PCR (Fig. 1B). For statistical analysis, protein fractions were classified as albumin, α1- (α1), α2-globulin (β), and γ-globulin (γ). Subclassifications of α and β fractions were not analyzed because adequate differentiation between α1- and α2-globulin or β1- and β2-globulin fractions was lacking in several of the samples. Intrarun reliability coefficients were excellent (>0.90) for all measured values except for total albumin (ALB mg/ml) and γ mg/ml, which were fair to good (0.59 and 0.48, respectively).

Total protein (TP mg/ml) concentrations are presented in Figure 2A. Note that TP mg/ml was significantly lower in exposed turtles as compared to control turtles in the last sample collected prior to death (Last, P = 0.014).

The percentages of protein fractions separated using AGE in exposed and control RES are available in Supplemental Figure S1. There was a significantly larger decrease in ALB from Pre-exp to Viremia values in exposed turtles as compared to control turtles (P = 0.008; Fig. S1A). In exposed turtles, α1 was significantly lower at Last (P = 0.014; Fig. S1B) and β2 was
significantly higher at Viremia ($P = 0.024$; Fig S1C) as compared to controls. No significant difference between ranavirus-exposed and control turtles was detected in $\gamma_c$ (Fig. S1D).

Calculated protein fractions from electrophoretic analysis of the plasma samples and TP mg/ml measurements are presented in Figure 2. ALB mg/ml was significantly lower at Viremia and Last in exposed turtles as compared to control turtles ($P = 0.036$ and 0.036, respectively; Fig. 2B). There was a significant decrease in total $\alpha$ (ALB mg/ml) at Last in exposed turtles as compared to control turtles ($P = 0.002$; Fig. 2C). Significant differences in $\beta_{mg/ml}$ or $\gamma_{mg/ml}$ between groups were not observed (Fig. 2D,
Table 1. A comparison of the percentage of frog virus 3–like ranavirus–exposed or control red–eared sliders with Viremia and Last plasma protein measurements outside of limits of pre-exposure ranges. Only values with significant differences between ranavirus‐infected and control groups are shown.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Plasma protein fraction</th>
<th>Limit of pre-exposure result</th>
<th>% of ranavirus‐exposed turtles (n = 4)</th>
<th>% of control turtles (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viremia</td>
<td>Albumin (mg/ml)</td>
<td>&lt;9.5</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>β-globulins (%)</td>
<td>&gt;20.9</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Albumin/globulins</td>
<td>&lt;0.71</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>Last</td>
<td>Total protein (mg/ml)</td>
<td>≤21.4</td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Albumin (mg/ml)</td>
<td>&lt;9.5</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>α-globulins (%)</td>
<td>≤21.9</td>
<td>100</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>α-globulins (mg/ml)</td>
<td>≤6.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

E, respectively). The albumin to globulin (A : G) ratio was significantly lower at Viremia in exposed turtles as compared to control turtles (P = 0.049; data not shown).

No significant differences in protein concentrations were noted between the exposed and control groups at Pre‐exp or Post‐exp. Pre‐exp data from all turtles were combined to illustrate protein concentration intervals in healthy RES maintained in a controlled, 22°C environment (Fig. S2). Because of the low number of individuals in this study, a rigorous evaluation of sensitivity and specificity of plasma protein subsets is inappropriate. However, an evaluation of which individuals were outside the Pre‐exp ranges reveals some interesting findings. The relevant limits are indicated on Figures 2 and S1 as dashed horizontal lines for the protein fractions, with significant differences between FV3‐like virus–infected and control turtles.

At two time points, Viremia and Last, a larger percentage of infected than uninfected RES had specific plasma protein values outside of the Pre‐exp range (Table 1). At Viremia, ALBmg/ml lower than 9.5 mg/ml was observed in three of four exposed turtles and zero of eight control turtles (Fig. 2B); βg values above 20.9% were observed in three of four exposed turtles and two of eight control turtles (Fig. S1C); and three of four samples from exposed turtles had an A : G ratio <0.71, whereas two of eight samples from control turtles were below this value. In samples taken just prior to death (Last), TPmg/ml in two of four exposed turtles and one of eight control turtles was lower than the Pre‐exp range of 21.4 mg/ml (Fig. 2A); αg below 21.9% was observed in four of four samples from the exposed group and one of eight samples from control turtles (Fig. S1B); and all Last samples below the αmg/ml Pre‐exp range of 6.0 mg/ml were from exposed turtles (Fig. 2C).

DISCUSSION

The TPmg/ml as measured by a dye binding (Bradford) assay in this study correlates with the previously published total solid concentration as measured by refractometry (r = 0.70). Both measurements observed a significant decrease in plasma protein concentrations of FV3‐like virus–infected RES prior to death. Additional changes were detected in this study 1–4 days prior to death using plasma AGE: namely, there were significant decreases in ALBmg/ml, αg, and αmg/ml. These changes are consistent with loss of protein associated with the vasculitis noted histologically in these individuals, and suggest that one of the major disease processes of FV3‐like virus infection involves third spacing of body fluid and circulatory collapse in RES. However, free coelomic fluid was not observed at necropsy, and a significant increase in βg was observed at an earlier time point (Viremia), indicating that vasculitis was not the only mechanism causing decreased ALBmg/ml in these turtles. In light of changes found in acute‐phase protein transcripts in Chinese soft‐shelled turtles infected with A. hydrophila, lack of protein production by hepatocytes is likely a contributing factor to the changes in plasma protein concentrations observed in this study.

A classic mammalian acute‐phase protein response would include a decrease in albumin and an increase in α, β, and/or γ fraction concentrations. Observational studies in cheloniids suggest that decreases in all protein electrophoresis fractions occur during an acute‐phase protein response in turtles. In this study, there was a significant decrease in ALBmg/ml and increase in βg, but only a mild decrease in TPmg/ml at Viremia. A significant decrease in TPmg/ml, ALBmg/ml, βg, and αmg/ml was observed just prior to death. These changes suggest that an acute‐phase protein response is occurring in ranavirus‐infected RES.
No significant changes were observed in the \( \gamma \) values during the course of this study. The \( \gamma \) fraction in reptiles is primarily (although not exclusively) comprised of immunoglobulins. The lack of change in the \( \gamma \) fractions of ranavirus-infected turtles is not unexpected given the classically slow seroconversion process of reptiles and the naïve status of the experimental individuals.\(^6,29,44\) Reevaluation of this particular facet of the acute-phase protein response would be appropriate in RES that had recovered from ranavirus infection or in RES afflicted with a more indolent disease process.

The evaluation of plasma protein fractions by the number of individuals outside the Pre-exp range reveals interesting findings that suggest a clinically relevant use of these variables as a diagnostic tool. It must be remembered when considering these data that they are derived from a small number of individuals and a rigorous evaluation of sensitivity and specificity is inappropriate. Additionally, it is well accepted that all analytical processes will have a degree of inherent error and that any diagnostic tool should be evaluated for observed error.\(^40\) Measurement of plasma protein fractions in additional healthy and ranavirus-infected RES is needed to determine the predictive value of these findings in a clinical setting and produce appropriate recommendations for total allowable error.

Electrophoretic patterns of plasma proteins from a variety of turtle species have previously been published revealing species-specific patterns in plasma electrophoretograms.\(^4,11,33,37\) This has led to the practice of speciating turtles based on their electrophoretic and protein profiles. It is interesting to note that several of the published species-specific profiles are more consistent with the ill RES than the healthy control group in this study. One excellent example can be seen in eight specimens of the European pond turtle, *Emys orbicularis*, presented by Musquera et al.\(^33\) It is possible that individual animals used in some of the previous studies were not healthy; however, it is more likely that knowledge of the species-specific normal electrophoretic and protein profiles is required before using plasma electrophoresis to screen for an acute-phase protein response in reptiles.\(^41\)

Comparing the observed mean values from the Pre-exp group to reference intervals published for RES, it is noted that the TP\(\text{mg/ml} \) and \( \alpha \), \( \beta \), and \( \gamma \) fractions. Differences in staining techniques and manual band assignments may have led to differences between studies. Third, seasonal differences, independent of temperature, affect innate and adaptive immunity in wild RES.\(^44\) It is possible that seasonality affected values in the captive RES used in this study. Finally, environmental temperature affects ectotherm protein production and chelonian immunoglobulin production.\(^29,44\) As part of this current study, turtles were intentionally housed at a suboptimal environmental temperature, which may have resulted in differences between studies.

The results of this study should be viewed as representative of response in suboptimal, yet commonly encountered, environmental conditions, as turtles in this study were maintained in an environment that prevented them from attaining their preferred internal temperature range of 24–30°C for healthy adult RES.\(^3,13,30,36\) Multiple studies have shown that, at suboptimal temperatures, ectotherms have an altered metabolism and immune response. Marked differences in phagocytic ability, complement activity, immunoglobulin levels, and delay in seroconversion are observed at different temperatures in multiple species.\(^6,29,31,32,38\) The acute-phase protein response should be investigated at normal and potentially elevated temperatures to better understand the plasma protein changes as a function of temperature in RES.

Significant changes were noted in plasma AGE data of FV3-like virus–infected turtles at two time points: Viremia (7–21 days prior to death) and Last (1–4 days prior to death). These changes suggest that a decrease in ALB\(\text{mg/ml} \) with an increase in \( \beta_{\text{g}} \) may be an early indicator of inflammatory disease in RES. Also, decreases in TP\(\text{mg/ml} \), ALB\(\text{mg/ml} \), \( \gamma_{\text{g}} \), and \( \alpha_{\text{mg/ml}} \) may be observed 1–4 days prior to death due to ranavirus infection in RES. This study provides information needed to critically evaluate the plasma protein response in RES and helps to confirm the existence of an acute-phase protein response during a known inflammatory disease.

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LITERATURE CITED


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