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Comparison of complement system activity amongst wild and domestic animals

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Abstract. Multiple mechanisms have evolved for the defensive recognition of foreign components, such as microorganisms. The majority of immunological studies with vertebrates have been focused on endothermic species, and relatively little attention has been directed toward ectothermic vertebrates. We employed a colorimetric assay designed to compare plasma hemolytic activities based on the serum complement system (CS) activities amongst some representative reptiles, wild and domestic birds, and mammals. Results obtained from the hemolytic assays conducted with plasma derived from all of the animal species used showed that broad-snouted caiman had the highest activity, and no differences were observed in the hemolytic activities of plasma from birds or the other reptile species. In contrast, the CS activity obtained with mammalian plasma was markedly lower than that from the other taxa. This assay has many advantages, such as the requirement of small sample volume, reproducible results, and low cost. In addition, unsensitized sheep red blood cell hemolysis can be successfully used for the evaluation of innate immune system activities in non-mammalian species; however, for mammals, it should be combined with other immunological determinates to evaluate integral innate immunocompetence.

Keywords. Innate immunity, immunocompetence, complement system, wildlife, domestic animals.

Organisms are continually exposed to a multitude of pathogens through contact, ingestion, and inhalation. Multiple mechanisms have evolved for the recognition of foreign antigens such as microorganisms. These strategies are the result of multiple cascade events that converge in the release of molecular signals that stimulate the recognition of foreign antigens. Those mechanisms are strategically divided in two distinct, but related, systems: acquired immunity and innate immunity. The innate immune system also plays a critical role in priming and stimulating the adaptive immune response (Medzhitov and Janeway, 1997). The concept of innate immunity refers to the first line host defense that serves to restrain infection in the early hours after exposure to microorganisms (Hoffmann et al., 1999). In turn, the adaptive immune system, more complex in nature, activates innate effector mechanisms in an antigen-specific manner as a second line of defense. The connections between the various immune components are not fully understood, but recent progress has brought us closer to an integrated view of the immune system and its function in host defense.

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The majority of studies on vertebrate immunity have focused on endothermic species, and relatively little attention has been focused on ectothermic species (reviewed in Juul-Madsen et al., 2008; Hamon and Quintin, 2016). These species are not commonly studied because the features that control their growth, reproduction and general physiology are largely unknown. However, some studies have shown that the complement system, as part of the innate mechanism of fish and other poikilothermic vertebrates, is more diverse than that of higher vertebrates, and thus a broader range of antigens can be recognized (Sunyer et al., 1998).

Crocodilians exhibit well-characterized social behaviors and responses to stressors that can trigger serious disputes between co-specific species, predators, and even conflicts with human activities. As a result, they sometimes exhibit physical trauma, serious injuries and even the loss of entire limbs. Frequently, these animals live in environments, either natural or captive, containing a high concentration of potentially pathogenic microorganisms. In most cases, crocodilians tolerate these circumstances without showing signs of infection (Siroski et al., 2010).

The colorimetric assay used in this study detects and characterizes the serum complement system (CS, Merchant et al., 2006). It is based on the disruption of sheep red blood cells (SRBC) by immunological proteins circulating in plasma. These proteins recognize SRBCs as foreign antigens and initiate activation of the CS cascade, which culminates in the formation of a protein complex that generates a pore (membrane attack complex, MAC) in the SRBC membrane and its subsequent lysis. Upon lysis, released hemoglobin is quantified using a spectrophotometer and considered proportional to the CS activity. This assay is routinely used in clinical laboratories to assess CS activity (Nagaki et al., 1980).

To compare plasma hemolytic activity amongst different species of vertebrates, we collected blood samples from reptiles, wild and domestic birds, and mammals. Blood samples from juvenile broad-snouted caiman (*Caiman latirostris*; n = 8) were obtained from the spinal vein; from tegu lizard (*Salvator merianae*; n = 6), lagoon turtle (*Phrynops hilarii*; n = 5) and painted turtle (*Trachemys dorbigni*; n = 6) from the caudal vein. Blood samples were obtained from wild pochard ducks (*Netta peposaca*; n = 5), domestic ducks (*Anas domesticus*; n = 5), and swan geese (*Anser anser domesticus*; n = 6) from the brachial vein. Blood of following mammals were obtained from the jugular vein: maned wolves (*Chrysocyon brachyurus*; n = 4), spider monkeys (*Ateles chamek*; n = 5), and horses (*Equus caballus*; n = 5). All blood samples were collected using heparinized syringes. All animals appeared healthy, and none were undergoing antimicrobial treatment. Plasma was separated within 1 h of collection by centrifugation at 1500x*g* for 30 min, and stored at -18°C until analysis.

The hemolytic assay was adapted and performed to evaluate the hemolytic properties of serum CS activity from different animals. As mentioned above, the SRBC hemolysis assay is based on the hemolytic disruption of SRBCs by means of serum immunological proteins (Merchant et al., 2005; Merchant and Britton, 2006; Merchant et al., 2009; Siroski et al., 2010). Fresh SRBCs were obtained from heparinized whole blood collected from the jugular vein of Merino sheep (*Ovis aries*). Whole blood from sheep was washed several times with phosphate-buffered saline (PBS, pH 7.4) until the supernatant was clear, and then a 2% SRBC (v/v) solution was prepared (Siroski et al., 2010).

To make a comparison between the hemolytic properties of plasma caused by the serum CS from different animals against SRBC, each sample was treated independently. Validation assays were performed with the plasma of each species with and without ethylenediaminetetraacetic acid (EDTA) and mild heat treatment (56°C for 30 min), both considered as classical inhibitors of the CS (Morgan, 2008). The tests were conducted at laboratory ambient temperature (25°C ± 2°C) by placing 0.5 ml of plasma from each animal together with 0.5 ml SRBC (2% v/v in isotonic saline). After 30 min incubation, the mixture was centrifuged and 300 µl of supernatant was transferred to a well of a microtiter plate for analysis on microplate reader at 540 nm.

A positive control for hemolysis was obtained by 1% SRBCs solution and 0.1% (v/v) Triton X-100. This mix was aggressively injected and ejected several times through a tuberculin syringe until complete hemolysis was confirmed with an optical microscope (Olympus BH-2, Tokyo, Japan) at 400×. Optical density of the supernatant was measured in a microplate reader at 540 nm, and was considered to be maximum hemolysis. The samples were performed in quadruplicate to obtain valid statistical evaluations and results were expressed as the percentage of maximum hemolysis (MH%; mean ± standard error). Statistical analysis was performed using SPSS 16.0 software (SPSS for Windows 2007). Data were tested for normality with the Kolmogorov-Smirnov test, and homogeneity of variances between groups was verified by the Levene test. One-way analysis of variance (ANOVA) and Tukey's test were used to test for differences among groups, and a P value of 0.05 was considered statistically significant.

The hemolytic method based on the rupture of sheep red blood cells was very effective at estimating complement activity in every species tested. Plasma samples

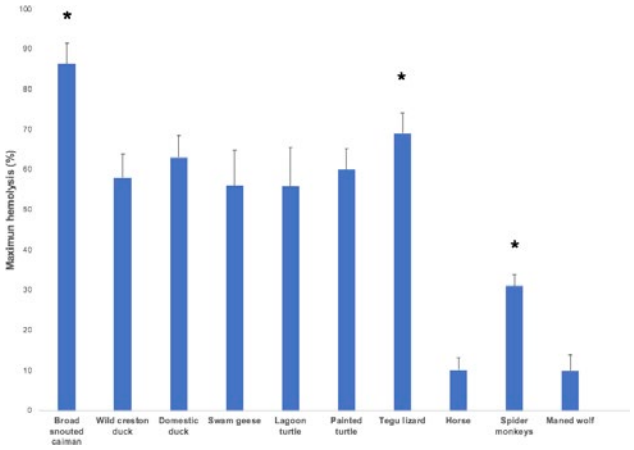

Fig. 1. Results obtained from the evaluation of the complement system activity from reptiles, wild and domestic birds and mammals. * Indicates significant differences.

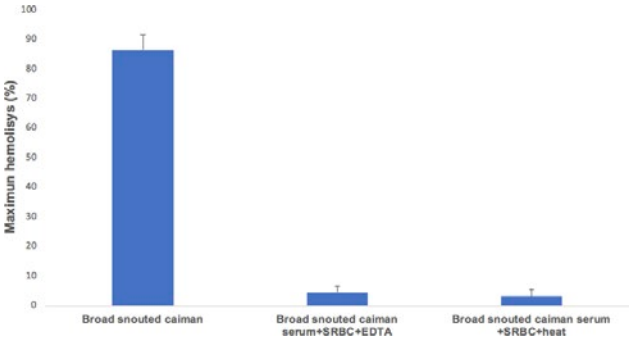

Fig. 2. Results obtained from the evaluation of the complement system activity from Broad-snouted caiman serum+SRBC with EDTA and heat.

from all species responded to the exposure to SRBC solution (2%). Results obtained from the hemolytic assays of plasma derived from ten species of vertebrates showed that *C. latirostris* plasma (86.38 ± 5.1) had the highest activity ($P < 0.05$; Fig. 1). No differences were observed in the %MH generated with plasma from birds and reptiles other than the caiman. However, the tegu lizard had the second highest %MH (69 ± 5.2) after caiman. There were no significant differences between %MH of domestic duck, swam geese and wild pochard ducks (63 ± 5.6, 56 ± 8.9 and 58 ± 5.9, respectively). Conversely, the %MH obtained with mammalian plasma was markedly lower than those from birds and reptiles. While the spider monkey exhibited 31 ± 2.9 activity, the maned wolf and horse had very low values of %MH (10 ± 3.9 and 10.2 ± 3, respectively).

In order to eliminate the possibility that the observed hemolysis was mediated by other hemolytic mechanisms, the CS assay included 2 classical inactivators of the serum complement, mild heat treatment of the serum and ethylenediaminetetraacetic acid (EDTA) (Morgan, 2008). Untreated serum, preheated serum (56°C for 30 min), and serum treated with 50 mM EDTA were exposed to 2% (v/v) SRBCs. Data demonstrated the ability of serum to rupture SRBC membranes (Fig. 2). In this case, %MH values of SRBCs with EDTA (4.4 ± 2.11) and heat (3.2 ± 2.17) were compared. The absorbance recorded indicated that there were significant reductions in the maximum hemolysis of SRBCs when complement-system inhibitors were added ($P < 0.001$; Fig. 2).

The hemolytic activities found for *C. latirostris* in this work were similar and comparable to that values previously reported from other crocodilian species, such as *Alligator mississippiensis* (Merchant et al., 2005), *Crocodylus porosus* and *Crocrodylus johnstoni* (Merchant and Britton, 2006) and *Crocodylus acutus* (Merchant et al., 2010). These data provide evidence that the mechanisms of complement activities for diverse crocodilian species are similar. The differences between the %MH values of each species is a consequence of different habitat where they live and therefore exposure to the large variety of pathogens.

The greater plasma hemolytic capacity of *C. latirostris* compared with that of turtle plasma had already been indicated by Ferronato et al. (2009) for *Phrynops geoffroanus*, who suggested that the higher activity detected in crocodilian species might be due to evolutionary pressure selection on this group because their environment is rich in potentially pathogenic microorganisms. The same reasoning might be extended to the species *S. merianae*, which showed high values, similar to those of turtles, but lower than that obtained for *C. latirostris*. In addition, it is interesting the fact that the values observed in these other reptilian species are lower than that observed for crocodilian species. The high complement activity of *S. merianae* could be associated with the aggressive behaviors displayed toward member of their own species. These conspecific aggressions may have been compensated by the evolutionary development of potent innate immunity (Merchant et al., 2010).

Birds have been shown to have a potent nonspecific immune mechanism based on the alternative pathway of CS activity. Mekchay et al. (1997) reported a significant hemolytic capacity in the plasma of birds and reported higher complement activities in wild birds than in captive birds raised under intensive commercial breeding conditions. These results are consistent with those previously detected by Skeeles et al. (1988) that chickens reared for human consumption exhibited lower CS activities compared to wild turkeys. Differences could be related to the

genetic composition of these birds or reflect a difference in the environment in which the chickens were raised. However, the results from our study revealed no differences between species of domestic and wild birds. The antimicrobial activity of one species of commercial poultry (*Gallus gallus*) was high, similar to that of reptiles, possibly resulting from a common evolutionary origin and developed in response to periodic exposure to a significant amount of pathogens (Siroski et al., 2010).

The highest hemolytic capacity among the plasma samples derived from mammals was detected in spider monkeys but was still much lower than that of non-mammalian species. Similarly, in a study conducted to determine levels of serum hemolysis in the plasma of nine primate species against sensitized and desensitized SRBC, the results were varied, but the plasma from primates mostly caused only a slight hemolysis of unsensitized SRBC (Ellingsworth et al., 1983). Other determinations were performed with camel (*Camelus dromedarius*) serum where lytic capacity was evaluated against desensitized erythrocytes from different species. They found that the erythrocytes in homologous species (goats, sheep, rat and bovine) were resistant to lysis, while the effect on heterologous erythrocytes was attributed to the presence of the alternative pathway (Olaho-Mukani et al., 1995). Similar findings were previously reported by Arya and Goel (1992) in buffalo (*Bubalus bubalis*) serum. In a study focused on the complement-mediated disruption of erythrocytes from 18 species of mammals and birds, it was observed that erythrocytes were not lysed by homologous complement, with the exception of guinea pig complement, which weakly lysed homologous erythrocytes, but with only 37% lysis maximum (Ish et al., 1993).

The low hemolysis values of erythrocytes exhibited by mammalian serum suggests that this restriction might be involved in the regulatory principle of non-specific and role-specific factors (Van Dijk et al., 1983). From these studies we can assume that plasma of some mammals did not hemolyze the SRBCs because they were not recognized as foreign and thus the mammalian CS was considered to recognize more limited range of antigens to trigger activation.

Although the main objective was to evaluate the differences between the activities of CS in the plasma of the various species studied, it is important to note that the assessment of immune function in wildlife has become an important tool for the investigation of ecological and evolutionary processes. The variety of tests that can be used in wild animals are often limited by the difficulty of capture and handling, and also difficulties of recovery or recapture, lack of specialized specific reagents, and small sample sizes of the study species (Matson et al., 2005). In this case, the assay employed had many advantages, such as the requirement of small sample volume, reproducible results, and low cost for the assessment of immunocompetence. Hemolysis of SRBCs has been used to assess the serum complement activity of crocodilians (Merchant et al., 2005; Merchant and Britton, 2006; Merchant et al., 2010; Siroski et al., 2010; Merchant et al., 2013a, 2013b), varanids (Merchant et al., 2012), snakes (Baker and Merchant, 2018), turtles (Ferronato et al., 2009; Baker at al., 2019), and amphibians (Major et al., 2011). The results showed that the test of unsensitized SRBC hemolysis can be used successfully for the evaluation of the innate immune system in a wide variety of species of reptiles and birds, but for mammals it should be utilized with other immunological determinations. These findings may reflect underlying differences in the biology and life history of each species.

Our study confirmed that the hemolytic method was very effective and advantageous at estimating complement activity in every species tested because small plasma samples from all species responded to the exposure to SRBC solution (Merchant et al., 2006). Furthermore, this assay allows to be routinely used in clinical laboratories to assess CS activity. Through the use of this hemolytic assay, we demonstrated a greater plasma hemolytic capacity of *C. latirostris* compared with other reptiles, birds and mammals. This means that the higher activity detected in crocodilian species might be due to evolutionary pressure selection on this group because their environment is rich in potentially pathogenic microorganisms and they exhibit aggressive territorial defense behaviors. Due to the close phylogenetic linkage with the reptiles, we built similar deductions with the group of birds.

Finally, it is important to highlight that the use of hemolytic method in the current study offered a contribution to the knowledge of comparative immunology. Also, it could be an appropriate tool to evaluate the role of complement in the immune function for some other wildlife species other than reptiles and for the investigation of ecological and evolutionary processes.

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tina). All animals were handled according to the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (CONICET, 2005). Samples were collected from captivity wild and domestic animals, which were maintained in a registered zoo by a National Resolution.

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