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Plasma Activity of the Broad-snouted Caiman (*Caiman latirostris*)

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Pablo A. Siroski, Carlos I. Piña, Alejandro Larriera, Mark E. Merchant, and Jose Di Conza (2009) Plasma activity of the Broad-snouted Caiman (*Caiman latirostris*). *Zoological Studies* **48**(2): 238-242. Crocodilians exhibit well-defined social behaviors, which frequently result in serious wounds as a consequence of social disputes including the loss of entire limbs. Despite the severity of many wounds, there is typically little sign of infection. A common question is how these animals survive with serious wounds without showing obvious signs of illness, particularly when living in environments containing potentially pathogenic microbes. In this study we determined *in vitro* plasma antibacterial activity of the Broad-snouted caiman (*Caiman latirostris*) against *Escherichia coli* and compared it to that in hen (*Gallus gallus*) and human plasma. Colony forming units were measured at different exposure times (0, 1, 3, and 6 h). The antibacterial activity of Broad-snouted caiman plasma was consistently superior to those of human and hen plasma, and hen plasma had greater activity than human plasma except at 3 h of exposure. Only *C. latirostris* plasma completely inhibited *E. coli* proliferation at 6 h. http://zoolstud.sinica.edu.tw/Journals/48.2/238.pdf

Key words: Crocodylia, Immune system, Broad-snouted caiman, Antibacterial activity, *Caiman latirostris*.

Immunity of animal species has evolved from innate mechanisms to more complex and adaptable immune responses designed to recognize materials unknown to them. The innate immune system also plays a critical role in priming and instructing the adaptative immune response (Medzhitov and Janeway 1997). The concept of innate immunity refers to the 1st line host defense that serves to restrain infection in the early hours after exposure to microorganisms (Hoffmann et al. 1999). It is widely known that blood contains important elements that mediate rapid responses to infection (Levy 2000). 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, so a broader range of antigens can be recognized (Sunyer et al. 1998).

Crocodilians exhibit well-defined social behaviors, which frequently result in serious wounds as a consequence of social disputes (Webb and Messel 1977, Webb and Manolis 1983, Piña 2002) including the loss of entire limbs. Despite the severity of many wounds, there is typically little sign of infection. A common question is how these animals sometimes survive such serious wounds without showing obvious signs of illness, particularly when living in environments containing microbes that can potentially cause infection. The antimicrobial activity of crocodilian blood was

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previously described (Britton et al. 2002, Merchant et al. 2003 2005a, Siruntawineti et al. 2004).

Reptiles, birds, and mammals are amniotes and, while reptiles have remained poikilothermic, birds have evolved into endothermic organisms. The immunological defense systems of all of these vertebrates share similarities (Rose 1979). In addition, poultry and crocodilian farms are similar in several aspects. Animals are exposed to stressful factors such as crowding and non-hygienic contaminated conditions, but they generally show limited, if any, ill effects. This suggests that these animals have very potent and efficient immune systems. The main goals of this study were to evaluate the antibacterial activity of Broad-snouted caiman (*Caiman latirostris*) plasma against *Escherichia coli*, and compare it with those of an avian (*Gallus gallus*) and mammalian (human) species.

MATERIALS AND METHODS

The susceptibilities of microorganisms to antibacterial substances are often determined in the lab by measuring the inhibitory capacity of such substances. We used a standard *in vitro* bactericidal assay that measured the antibacterial activities of *C. latirostris*, *G. gallus*, and human plasma against *E. coli* ATCC 11105 at different time intervals. We selected *E. coli* because it is an intestinal microorganism (part of the normal flora that often is present without causing disease) and a causative agent of a variety of diseases in humans and animals (Doyle et al. 2006). It can also be found in contaminated liquids and objects that have been in contact with fecal material, such as water in crocodilian farm ponds.

Blood samples from juvenile 18 mo old Broad-snouted caimans (n = 16, 80-100 cm in total length) were obtained from the spinal vein (Tourn et al. 1994, Zippel et al. 2003). The caimans were obtained through the Proyecto Yacaré ranching program (Larriera and Imhof 2006). Hens (n = 4, 8 mo of age, 1.8-2 kg) from a commercial farm were bled by cardiac puncture. Hen and caiman eggs were incubated in artificial incubators and raised under temperature-controlled conditions, and juveniles were fed ad libitum. Peripheral blood was collected from human volunteers (n = 3) by venipuncture of the cubital vein. All subjects appeared healthy, and none was currently undergoing antimicrobial treatment. All blood samples were collected using 0.8 x 38 mm needles and heparinized 5 ml syringe. Plasma was separated within 1 h of collection by centrifugation at 1500 g for 30 min, and stored at -18°C until being analyzed. Samples were treated individually.

Escherichia coli ATCC 11105 was used for the antibacterial assays. *Escherichia coli* was preserved in 25% glycerol at -18°C. After suspending cells in Luria-Bertoni (LB) broth, the *E. coli* cultures were plated on nutrient agar (Britania® Argentina) and incubated at 37°C for 18 h until cells reached the mid-logarithmic phase.

E. coli bacteria were incubated with human and animal plasma. The *E. coli* colonies were suspended in a sterile saline solution and diluted to 0.5 McFarland turbidity units (approximately 10^8 cells/ml). After mixing, 100 μl of a bacterial suspension was added to 900 μl of a plasma sample. At different times (0, 1, 3, and 6 h), 100 μl of each serial dilution was spread on a nutrient agar surface in Petri dishes and incubated overnight at 37°C. Time 0 corresponds to the moment at which the plasma sample and *E. coli* solutions were mixed, and then immediately spread onto the plate. Plates containing 30-300 colony forming units (CFUs) were utilized for analyses. In order to monitor the validity of the incubation assays, a parallel control using only a sterile saline solution, instead of caiman serum, was conducted, but was not included in our results. Each experiment was performed in quadruplicate. CFUs were calculated by multiplying the number of colonies counted at each dilution by the dilution factor and by 10 (0.5 McFarland turbidity units).

Data are expressed as the mean ± standard error (SE). Statistical analyses were performed to compare CFU/ml values between species, replicas, and individuals at different times.

RESULTS AND DISCUSSION

The likelihood that microorganisms cause illness in crocodilians increases under stressful conditions (Shotts et al. 1972, Brisbin 1982, Franklin et al. 2003). Such stressful conditions include extreme temperatures, capture and restraint, increased population density, an inappropriate diet, etc. Crocodilians and others reptiles are exposed to such conditions more frequently when they are in captivity than when they are in the wild.

Some studies have reported that ectothermic animals have developed defense mechanisms such as protective flora or physical barriers (skin

and gut mucosa) which facilitate the recognition of a wide range of microbial molecules (Flajnik 1996, Brames 2007). Many studies have been carried out to detect antimicrobial activities in a number of products derived from animal and vegetable sources (Marshall and Arenas 2003). Previous work on immunity in ectotherms was intended to compare their resistance to bacterial pathogens to those of humans and other mammals (Manning and Turner 1976, Merchant et al. 2003).

Shaharabany et al. (1999) were the first to demonstrate antibacterial activities in tissues of *Crocodylus niloticus* and a variety of wild and domestic birds. Recent studies showed antimicrobial serum activity in other crocodilians (Merchant et al. 2003 2004 2005a b, Siruntawineti et al. 2004, Merchant and Britton 2006). In this study, antibacterial activity against *E. coli* ATCC 11105 was detected in all plasma samples, but different sensitivities were shown depending on the species and time (Fig. 1).

Caiman latirostris plasma exhibited time-dependent inhibition of *E. coli* (Fig. 1). At the same time, the caiman's antibacterial activity was consistently superior to those of human and hen plasma ($p < 0.001$). Only caiman plasma had completely inhibited *E. coli* growth at 6 h of exposure, thus showing rapid bactericidal activity. Human plasma exhibited less of an effect than hen plasma on bacteria viability, except at 3 h of exposure, at which time, there was not significant difference ($p < 0.001$). There were no differences in antibacterial activities between replicas or individuals ($p > 0.05$) for any species. Similar results were obtained by Merchant et al. (2003) for *A. mississippiensis* and by Siruntawineti et al. (2004) for *C. siamensis*. Hen plasma showed a similar ability to kill *E. coli* at 6 h of exposure as caiman plasma, but human and hen samples were never able to completely inhibit *E. coli*.

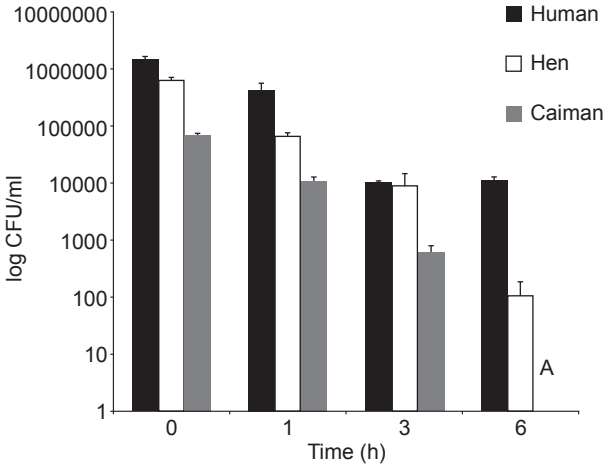

Fig. 1. Time-dependent (h) antibacterial action of *Caiman latirostris*, *Gallus gallus*, and human plasma exposed to *Escherichia coli*. Serial dilutions of each solution were plated to determine the colony-forming units (CFU)/ml. A, none detected.

Taylor (1983) reported on many studies examining bactericidal activity, but the data generated were often conflicting. Merchant and coworkers (2006a) described the advantage of a colorimetric assay to measure serum complement activity utilizing the hemolysis of sheep red blood cells. This method is very useful for detecting complement system activity, but it does not address other causes of antimicrobial activity. The antibacterial assays used in this work were a simple but valid method for studying plasma antimicrobial activity independent of bactericidal origin. However, it is not appropriate to compare our values with other similar studies in other species because each one used a different methodology (Merchant et al. 2003, Siruntawineti et al. 2004).

Caiman and hen plasma samples exhibited more dramatic and rapid *in vitro* response mechanisms than that of human plasma when exposed to *E. coli*. When in captivity, animals are exposed to immunological challenges by a great number of pathogens, including the species we tested. Initially, antimicrobial properties of crocodilians studied were attributed to peptides (Shaharabany et al. 1999) that exhibit antimicrobial activity, but it was also reported that they might also be attributed to the serum complement protein system (Merchant et al. 2003 2004 2005a b 2006a b). Many researchers have documented the presence of serum complement system in reptiles (Koppenheffer 1987, Sunyer et al. 1998, Zarkadis et al. 2001). Mastellos and colleagues (2004) described the complement system as a phylogenetically conserved arm of innate immunity, functioning together with the adaptive immune response by serving as an important inflammatory mediator of antigen-antibody interactions. Merchant et al. (2006b) found that extracts of *A. mississippiensis* leukocytes express cationic peptides with antimicrobial properties. According to those reports, antimicrobial properties of crocodilian tissues could have multiple origins.

Crocodilians represent an extremely successful group of organisms that have changed little for millions of years. These animals are sources of important information for those investi-

gating breakthroughs in immunology from a phylogenetic viewpoint. Animals preserve their unique individuality by distinguishing between self and non-self to protect against infection and possible extinction (Cooper 2002). Perhaps their capacity to resist attacks by microorganisms could be one of the reasons for their longevity.

The results of the present study confirm the presence of antibacterial activities in *C. latirostris*, *G. gallus*, and human plasma against *E. coli* (ATCC 11105). There was a time-dependent correlation between *C. latirostris* and *G. gallus* plasma bactericidal activity against *E. coli*. The results prove that the antibacterial activity of Broad-snouted caiman plasma toward *E. coli* is higher than those of hen and human plasma.

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