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Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*)

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Abstract

Treatment of alligator (*Alligator mississippiensis*) and human serum samples with *Escherichia coli* resulted in a time- and concentration-dependent inhibition of bacterial proliferation. When inoculated with *E. coli*, alligator serum exhibited 10-fold lower bacterial survival rates after 1 h than human serum. In addition, the antibacterial spectrum of alligator serum was shown to be much broader than that of human serum, with growth inhibition occurring in 100% of bacterial strains tested (compared to only 35% for human serum). Additional results showed that the antimicrobial activities of alligator serum could be completely inhibited by preincubation with proteases, indicating the proteinaceous nature of the antimicrobial activities. Furthermore, incubation of alligator serum at 56 °C for 30 min (classical human serum complement inactivation conditions) obliterated all antimicrobial properties of the alligator serum. The antibacterial activities occurred relatively quickly in vitro, with significant activity occurring within 5 min of inoculation with *E. coli* and maximal activity at 20 min. Also, the antimicrobial activity exhibited temperature dependence, with a substantial decrease in activity below 15 °C. These data suggest that the antimicrobial properties of alligator serum may be due to an active serum complement system.

Keywords: Serum; Antibacterial; Antimicrobial; *Alligator mississippiensis*; Crocodilian

1. Introduction

Eukaryotic organisms must continuously defend themselves against infiltration and colonization by microorgamisms. Host defense occurs via complex mechanisms and can be divided into two distinct, but interrelated, types of responses: acquired immunity and humoral (innate) immunity. Acquired immunity requires prior exposure to a specific antigen before a full immunological assault can be established by the host organism. In addition, the acquired immune response is complex and can often take several days to become fully activated. The humoral immune response comprises a significant portion of the immune system and acts as an initial defense mechanism against microbial growth shortly after infection occurs. These innate defense responses are activated shortly after exposure and act to slow or stop an infection in the initial stages so that the acquired immune response can be initiated. Also, the innate immune system functions in the activa-

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tion of the acquired immune system by generating chemotactic factors and producing cytokines that initiate the development and maturation of specific T-cell and B-cell populations.

Anecdotal evidence suggests that alligators are resistant to microbial infections. These animals often sustain serious injuries, including open wounds, due to interspecies fighting and collisions with boat propellers. However, despite the fact that they live in marsh environments, which may harbor potentially infectious microorganisms, alligators often heal without signs of infection. Despite the propensity of alligators to resist microbial infection, the mechanisms of immunity are not well characterized. The primary goal of this study was to investigate and characterize the antibacterial properties of the serum of the American alligator in vitro.

2. Materials and methods

2.1. Chemicals and biochemicals

Nutrient broth and nutrient agar were purchased from ISC Bioexpress (Kaysville, Utah). Lyophilized ATCC bacterial strains were purchased from Chrisope Technologies (Lake Charles, LA). The following ATCC-registered strains were used: *Klebsiella oxytoca* (49131), *Providencia stuartii* (33672), *Escherichia coliform* (25922), *Proteus mirabilis* (43607), *Enterobacter aerogenes* (49469), *Salmonella typhimurium* (14028), *Pseudomonas aeruginosa* (27853), *Citrobacter freundii* (C109820), *Shigella sonnei* (25931), *Shigella dysenterae* (13313), *Salmonella poona* (4840), *Yersenia enterocolitica* (9610), *Staphylococcus pyrogenes* (19615), *Streptococcus epidermitis* (19615), *Staphylococcus aureus* (6538), and *Enterococcus faecalis* (29212). Protease derived from *Streptomyces griseus* was purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Treatment of animals

Alligators were captured and housed at the Rockefeller State Wildlife Refuge in Grand Chenier, Louisiana. Numerous juvenile alligators, which were hatched in captivity from eggs collected in the wild, were maintained at Rockefeller Refuge in fiberglass-lined concrete tanks approximately 4.57-m long×1.83-m wide. Several small alligators (up to 91-cm in length) are housed in a single tank. Adult alligators were typically captured at night with use of a cable snare.

The environment in the tanks consisted of 50% dry bottom and 50% water of approximately 2.5-cm depth. The temperature is maintained at a constant 30–31 °C. The alligators are fed formulated dry pellets four times per week and the cages are cleaned five times per week. Blood samples were drawn from the supravertebral branch of the internal jugular vein (Olsen et al., 1977) using a heparinized 1.5 inch 18 gauge needle and a 10 ml syringe and transferred to either serum or plasma (50 mM EDTA) vacutainer tubes.

2.3. Bacterial cultures

Bacteria were maintained on nutrient agar slants at 4 °C. The day before an experiment, a 4 ml nutrient broth liquid culture was inoculated from the slant with a sterile cotton swab. The bacteria were allowed to incubate at 37 °C overnight to obtain log-phase culture. Serial dilutions of the log-phase cultures were plated on nutrient broth agar in 100 mm Petri dishes to determine the colony-forming units (CFUs) in each culture.

2.4. Determination of CFU

Fifty microliters of a dilution of each sample was spread onto the surface of nutrient broth agar plates to determine the CFUs for each sample. Samples were typically plated at three different dilutions to obtain plates with a quantity of colonies such to provide a reasonable estimate of bacterial density (50–400 CFUs/plate).

2.5. Concentration-dependent antibacterial properties of human and alligator sera

Two milliliter samples containing various concentrations of human or alligator sera in sterile 12×75 mm culture tubes were inoculated with approximately 1×10^5 *E. coli* bacteria from a log phase culture and incubated for 12 h at 37 °C. The optical density of each sample was measured at 0, 3, 6 and 12 h using the Varian Cary 50 spectrophotometer at 430 nm.

2.6. Antibacterial capacity determination

E. coli cultures in log growth phase were used to make 10-fold serial dilutions in sterile saline.

Fresh alligator or human sera samples (450 μl) were treated with 50 μl of bacterial culture or a dilution containing different amounts of bacteria. The samples were incubated at room temperature for 1 h and the CFUs for each culture was determined by the solid medium bacterial growth assay as described above.

2.7. Effects of serum on the growth of different strains of bacteria

Nutrient agar was dissolved in boiling water and 30 ml aliquots were transferred to 70 ml culture tubes, autoclaved at 121 °C and 18 psi (30 min) and held in liquid phase in a 45 °C water bath. The aliquots of sterile nutrient broth agar were inoculated with 100 μl of a log phase culture of one of various bacterial strains and then dispensed into 145×20 mm Petri dishes. After the agarose set, wells were cut with the large end of sterile, cotton-plugged Pasteur pipettes attached to a vacuum line. Twenty-five microliters of serum was pipetted into each well and allowed to incubate at room temperature for 3 h. Another 30 ml aliquot of sterile nutrient broth agar (45 °C) was poured onto the top of the original agar layer and allowed to set. The plates were incubated in an inverted position overnight at 37 °C and the zones of bacterial growth inhibition were measured. For slower growing species of bacteria, plates were incubated for 48 h.

2.8. Statistics and controls

All experiments were performed in quadruplicate to obtain valid statistical evaluation of the results. CFUs/ml for each sample were calculated by multiplying the number of colonies counted by the dilution factor and then by ten (due to the fact that only 50 μl were plated on each dish). Zones of growth inhibition were measured and the width of the well subtracted prior to statistical calculations. All results represent the means ± S.D.

3. Results

Inoculation of nutrient broth with approximately 10^6 CFUs of *E. coli* resulted in a time-dependent increase in bacterial proliferation as measured by spectrophotometry at 430 nm (Fig. 1a,b). Inclusion of different concentrations of human or alligator serum in the broth produced a concentration-dependent decrease in bacterial growth. Inoculation of 10% alligator serum reduced bacterial growth by 84% at 3 h, compared to only a 47% reduction by human serum (Fig. 1a,b). The addition of 10, 25, 50, 75 and 100% alligator serum resulted in statistically significant ($P<0.01$) decreases in bacterial growth at all time points after zero. Inoculation of 100% alligator serum that had been thermally inactivated (56 °C, 30 min) produced only a 16% decrease in growth as compared to 97% growth inhibition by serum that had not been heat-treated (Fig. 1c). Likewise, the growth of bacteria in 100% human serum that had been heat-treated showed only a 12% reduction in growth, relative to nutrient broth cultures (Fig. 1c).

Measurement of the antibacterial capacity of human and alligator serum (Fig. 2) revealed that alligator serum killed approximately 11- or nine-fold more bacteria than did human serum when samples were challenged with 10^4 CFUs/ml or 10^3 CFUs/ml, respectively. Inoculation of alligator serum with 10^5 CFUs/ml, 10^4 CFUs/ml or 10^3 CFUs/ml of *E. coli* resulted in a stepwise decrease in bacterial survival of 54%, 3% and 5%, respectively. In contrast, treatment of human serum resulted in 66, 44 and 44% survival for the same inoculations. Inoculation of cultures with 10^6–10^8 CFUs of *E. coli* overloaded the capacity of the serum complement to kill the bacteria, resulting in 100% bacterial survival in all three sample groups for both human and alligator serum. However, inoculation of serum with 10^5 CFUs/ml resulted in a 46% and 34% decrease ($P<0.01$) in bacterial survival for alligator and human serum, respectively.

Treatment of 16 different strains of bacteria (representing 13 different genera) with alligator serum resulted in 100% growth inhibition (Fig. 3). In comparison, human serum was only effective on 35% of bacterial strains. Human serum exhibited antibacterial activities toward only six of 12 gram negative strains and was completely ineffective against the four gram positive strains investigated. Furthermore, the degree of antimicrobial activity was significantly lower for all bacterial strains tested in human serum than that of the alligator.

The data in Fig. 4 reveal the kinetics of the in vitro antibacterial activities of alligator serum. After inoculation of alligator serum with 10^4 CFU *E. coli*/ml serum, antibacterial activity was detected (17% of *E. coli* killed) after 5 min. This

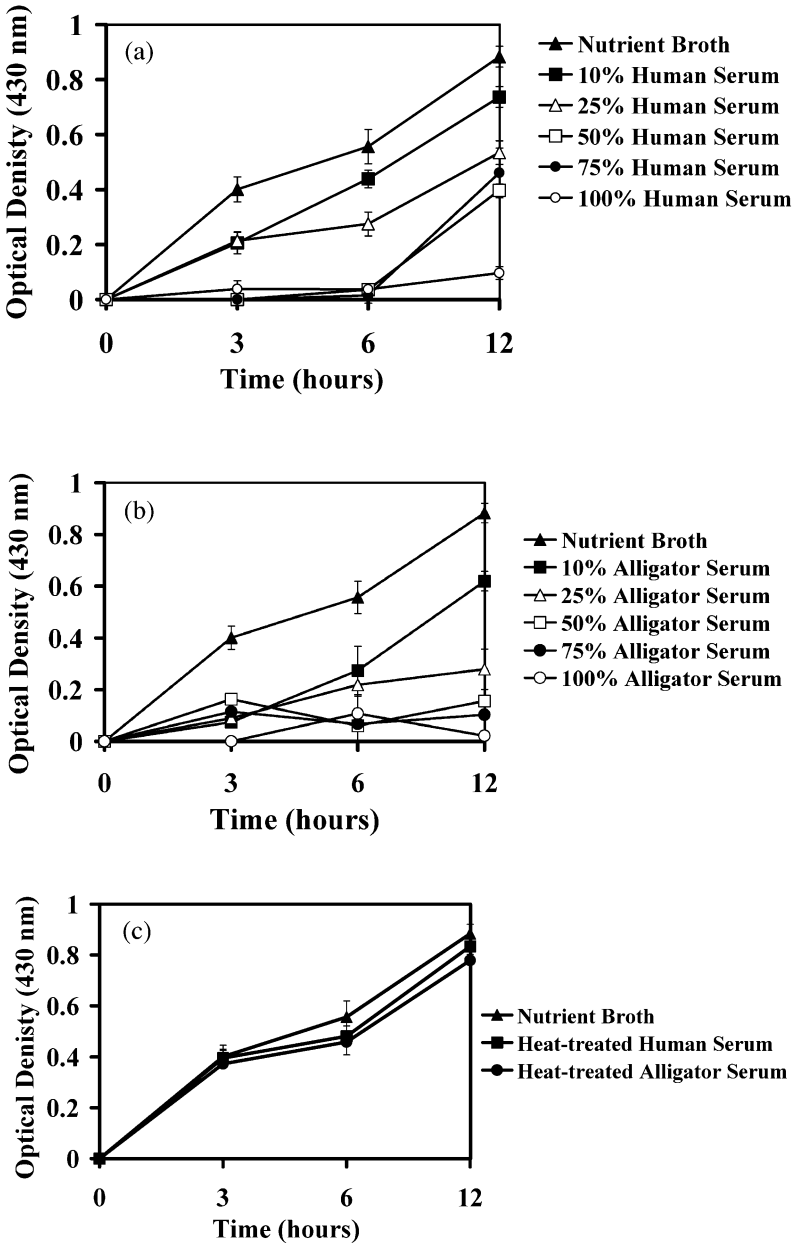

Fig. 1. Concentration-dependent inhibition of bacterial growth by human and alligator serum. Culture tubes containing 2 ml of 0, 10, 25, 50, 75 or 100% human (a) or alligator serum (b) or 2 ml of heat-treated (56 °C, 30 min) alligator or human serum (c) were inoculated with 10^5 CFU *E. coli*. The cultures were incubated at 37 °C and their optical densities were measured (430 nm) at 0, 3, 6 and 12 h post-inoculation. The results are expressed as the means ± S.D. for four determinations.

activity was statistically greater than saline-treated controls ($P<0.01$). The percentage of *E. coli* killed by the serum was increased to 43%, 62%, and 87% at 10 min, 15 min and 20 min post-inoculation, respectively. The antibacterial activity reached a maximum at 20 min and showed no statistically significant increases at 30 and 60 min.

The results displayed in Fig. 5 show the effects of temperature on the antibacterial effects of alligator serum. Inoculation and subsequent incubation (30 min) of serum samples at 5 °C and 10 °C resulted in a 76 ± 12% and 36 ± 9% reduction ($P<0.01$) in the effectiveness of the serum to kill *E. coli* bacteria, respectively. These results varied

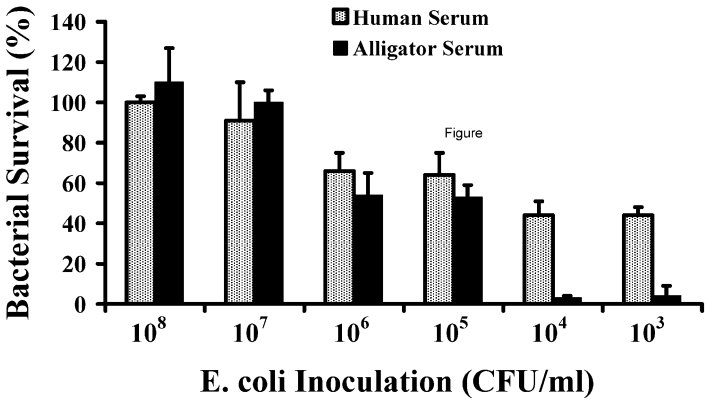

Fig. 2. Bactericidal properties of human and alligator serum. Serial dilutions of a log phase *E. coli* culture were added to 0.5 ml serum samples and incubated for 20 min. Dilutions of each sample were plated to determine CFUs. Results are expressed as bacterial survival ([CFUs after incubation/CFU added to sample]×100) and represent the means±S.D. for four determinations.

substantially from those obtained from the inoculation of nutrient broth control cultures at 5 and 10 °C. The chilled nutrient broth exhibited no effect on bacterial survival, which indicates that the antibacterial effects in the serum were not simply due to the low temperature incubation altering the growth of the *E. coli* cultures. Inoculation and incubation of serum samples at 15, 20 and 30 °C showed no reduction in antibacterial activity ($P>0.05$). However, inoculation and incubation of alligator serum at 35 °C resulted in a significant ($P<0.05$) 15.2% decrease in antibacterial activity. Furthermore, inoculation and incubation of serum at 40 °C resulted in a 30.8% decrease in activity.

Table 1 exhibits the effects of heat-treatment and preincubation of alligator and human sera with proteases on their antibacterial effects. Treatment of human or alligator sera for 30 min at 56 °C completely obliterated antimicrobial activities, as determined by a solid phase growth inhibition assay as described in the Section 2. In addition, pretreatment of a 100 μl sample of human or alligator serum with 20 μl of a protease derived from *S. griseus* (Sigma Chemical Company) eliminated antimicrobial activities.

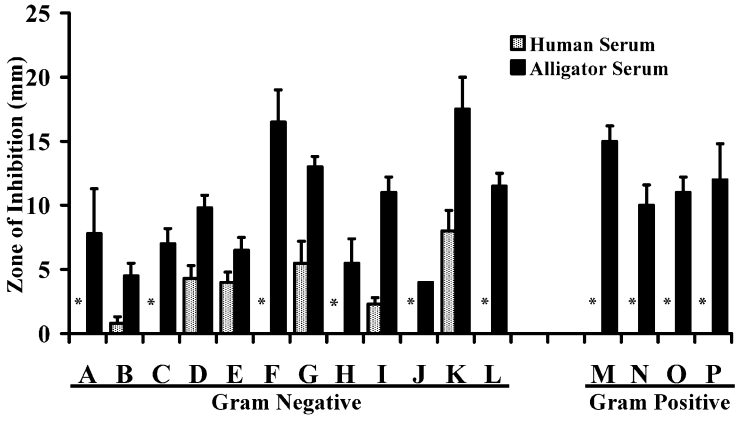

Fig. 3. Antibiotic spectrum of human and alligator serum. Aliquots of nutrient agar in liquid phase (45 °C) were inoculated with one of various strains of bacteria and poured into 125 mm Petri dishes. Samples of alligator or human sera were transferred into aseptically formed wells. After a 3 h incubation at room temperature, top agar was poured over the plates and the samples were incubated overnight at 37°C. A=*K. oxytoca*, B=*P. stuartii*, C=*Escherechia coliform*, D=*P. mirablis*, E=*E. aerogenes*, F=*S. typhimurium*, G=*P. aeroginosa*, H=*C. freundii*, I=*S. sonnei*, J=S dysenteriae, K=*S. poona*, L=*Yersinia enterocolitica*, M=*S. pyrogenes*, N=*Staphylococcus epidermitis*, O=*S. aureus*, P=*E. faecalis*. *=Not detected. The results are expressed as the means ±S.D. for four determinations.

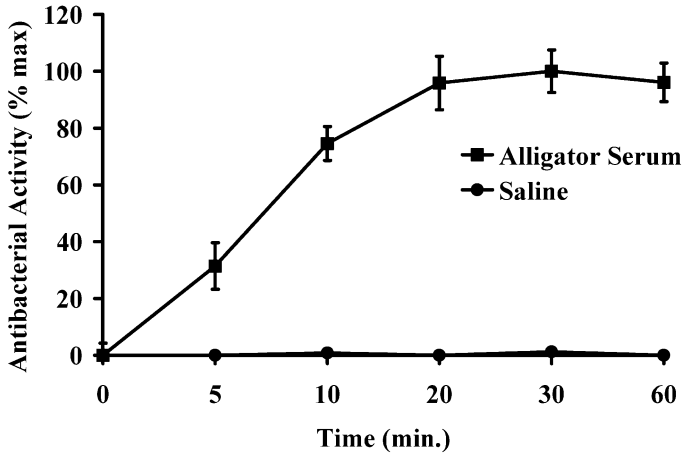

Fig. 4. Kinetics of the antibacterial action of alligator serum. Serum samples (1 ml) were inoculated with 10^4 CFU *E. coli*. At different time points after the inoculation, aliquots were removed, immediately diluted and plated on nutrient agar. After a 24 h incubation at 37 °C, the colonies were counted and the CFUs were determined. The results reported as percent survival and represent the means ± S.D. for four determinations.

4. Discussion

Exposure of eukaryotes to pathogenic microorganisms results in stimulation of complex host defense mechanisms. The spectrum of protective immunological mechanisms includes the induction of innate immune mechanisms. Innate immunity is non-specific in nature and requires no previous exposure to a specific antigen (Hoffman et al., 1999).

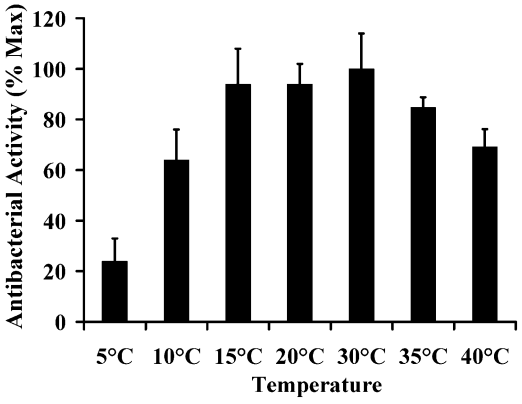

Fig. 5. Temperature-dependence of the antibacterial action of alligator serum in vitro. Serum samples (1 ml) were inoculated with 10^4 CFU *E. coli*. The samples were incubated at the indicated temperatures for 30 min, diluted and plated on nutrient agar. The results are expressed as the means ± S.D. for four determinations.

The immune systems of crocodilians have not been well-characterized. However, several reports have described the presence of cellular components of the immune system in the American alligator. Cuchens and Clem (1979a) have reported the presence of B-like and T-like lymphocytes in the alligator. In addition, Mateo et al. (1984a) described the morphological and cytochemical characteristics and relative abundance of various types of alligator peripheral blood cells. Huchzermeyer and Cooper (2000) reported the extrusion

Table 1
Effects of protease- and heat-treatment on the antibacterial activity of human and alligator serum

	Zone of growth inhibition (mm)
Human serum	
Untreated	2.3 ± 0.4
Heat-treated	ND
Protease-treated	ND
Alligator serum	
Untreated	6.5 ± 1.0
Heat-treated	ND
Protease-treated	ND

Twenty microliters of each serum sample were transferred to 6 mm wells cut into nutrient agar that had been inoculated with *E coli.* The samples were allowed to incubate in the wells for 3 h, nutrient top agar was poured over the surface and the plates were incubated overnight at 37 °C. The results are expressed as the means ± S.D. for four determinations. ND = not detected.

of fibrin and leukocyte infiltration into open wounds in the crocodile. Still other studies have provided morphological descriptions of the acute phase inflammatory reaction in alligators (Mateo et al., 1984b). However, no description of a humoral immune response has been reported in alligators.

Alligators seldom exhibit detrimental health conditions due to infection. In addition, alligators often sustain serious injuries and, despite the aqueous conditions in which they live, in many cases heal without infection. Crocodilians have been known to live with opportunistic pathogenic bacterial infections but often exhibit no physiological effects (Madsen, 1993; Madsen et al., 1998; Manolis et al., 1991). While crocodilians are not completely immune to microbial infections (Gorden et al., 1979; Novak and Seigel, 1986; Brown et al., 2001), these species do exhibit remarkable resistance to microbial colonization. The results from this study provide the first evidence that the American alligator has an active innate immune system. Sharbanay et al. (1999) have described the antibacterial activity in the serum of the saltwater crocodile. However, to our knowledge, a detailed functional characterization of a crocodilian innate immune system has not been published to date.

Human complement contains heat-labile proteins that are thermally inactivated when incubated at 56 °C for 30 min. The fact that the antibacterial activity of alligator serum can be obliterated under these conditions (Table 1) provides circumstantial evidence that these activities are due to the presence of a crocodilian complement immune system component. The concentration-dependent increase in antibacterial activity by alligator serum (Fig. 1) also suggests the presence of complement activity. This is not surprising considering that serum complement activity has been reported in other reptilian species (Kawaguchi et al., 1978; Koppenheffer, 1987). The inclusion of a thermally-inactivated 100% alligator and human serum controls demonstrates that the bacterial growth inhibition is due to the effect of a heat-labile serum factor(s) and not dilution of the nutritive value of the liquid growth medium by the addition of increasing amounts of serum.

The data listed in Table 1 provide evidence that the antimicrobial activities observed in alligator blood are due to the serum complement system of protein present in higher eukaryotes but not yet characterized in crocodilian systems. Incubation of alligator serum at 56 °C for 30 min (classical conditions for the inactivation of human complement) completely depletes the serum of antibacterial activities. In addition, pretreatment of serum with proteases also diminishes the antimicrobial properties indicating that these activities are dependent on the action of protein(s). The decrease in antibacterial activity observed for heat-treated alligator serum indicates that the antimicrobial activity is not due to antimicrobial peptides. Peptide-mediated antimicrobial activity is generally considered to be heat stable (Diamond et al., 1991; Moore et al., 1991).

Results from the investigation of the range of antibacterial activity demonstrated that alligator serum has a much broader spectrum of antibacterial activity than that of human serum (Fig. 3). Human serum complement proteins are relatively ineffective against gram positive bacterial strains, presumably due to the expression of proteins that inhibit complement function (Navarre and Schneewind, 1999). However, the alligator serum was highly effective as an antibacterial agent against all four gram positive strains tested (Fig. 3).

Alligator serum exhibits rapid (after only 5 min) antibacterial effects in vitro when challenged with *E. coli*. The effects occur rapidly as significant antibacterial activity was observed after the inoculation of alligator serum with 10^4 *E. coli* CFU/ml serum. This activity was statistically greater than saline-treated controls ($P < 0.01$). Increased antibacterial activity was observed at 10 and 15 min post-inoculation. Maximal activity was achieved at 20 min and did not increase at 30 and 60 min. The kinetics of the in vitro antibacterial effects of alligator serum on *E. coli* are almost identical to the in vitro actions of human complement on *E. coli* previously reported (Wright and Levine, 1981).

Since alligators are poikilothermic vertebrates, the effects of temperature on the antibacterial activities of alligator serum were investigated. Results from studies in which temperature probes were surgically implanted into the peritoneal cavities of wild alligators have revealed that internal body temperatures commonly range from 5 to 30 °C (Brisbin et al., 1982; Seebacher et al., 2003). The results in Fig. 5 demonstrate that the antibacterial effects of alligator serum are compromised at low temperatures in vitro. The antibacterial activities demonstrate temperature-dependent

decreases below 15 °C. These in vitro results may indicate that alligators may be immunocompromised during the winter months when body temperatures are often below 15 °C. The decrease in activity at temperatures above 30 °C is in agreement with reports that most physiological processes in the alligator are optimized near 30–31 °C (Lance, 1994; Glassman and Bennett, 1978; Cuchens and Clem, 1979b).

This study describes potent and broad-spectrum antibacterial activities in the serum of the American alligator. The fact that the antibacterial activities are heat labile, susceptible to protease activity, temperature-dependent and occur rapidly in a cell-free system suggests that these effects may be mediated by serum complement proteins. We believe that an active serum complement system may be partially responsible for the antibacterial properties of alligator serum.

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