J. Parasitol., 90(6), 2004, pp. 1480–1483

AMOEBACIDAL EFFECTS OF SERUM FROM THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*)

Mark Merchant, Damon Thibodeaux, Kara Loubser, and Ruth M. Elsey*

Department of Chemistry, McNeese State University, Lake Charles, Louisiana 70609. *e-mail: mmerchant@mcneese.edu*

ABSTRACT: Treatment of axenic *Naegleria gruberi* cultures with alligator serum resulted in time-dependent amoebacidal activity, with measurable activity at 5 min and maximal activity occurring at 20 min. The amoebacidal activity was concentration dependent, with measurable activity at 25% serum, whereas treatment of amoebas with undiluted serum resulted in only 16% survival. The efficacy was dependent on the concentration of amoebas, with higher survival rates at high amoeba densities and lower survival rates at low amoeba densities. The amoeba-killing effects of alligator serum were broad in spectrum because the serum was effective against 3 strains of *Naegleria* species tested and 4 *Acanthamoeba* species, which have been reported to be resistant to human serum complement–mediated lysis. The amoebacidal effects of alligator serum were temperature dependent, with optimal activity at 15–30 C and a decrease in activity below 15 C and above 30 C. The amoebacidal activity of alligator serum was heat labile and protease sensitive, indicating the proteinaceous nature of the activity, and was also inhibited by ethylenediaminetetraacetic acid, which indicated a requirement for divalent metal ions. These characteristics strongly suggest that the amoebacidal properties of alligator serum are because of complement activity.

Naegleria spp. and *Acanthamoeba* spp. include free-living organisms distributed widely in soils and freshwater habitats throughout the world (Marciano-Cabral, 1988; Marciano-Cabral and Cabral, 2003). Organisms from these genera are known to be opportunistic parasites (Martinez and Janitschke, 1985; Martinez and Visvesvara, 1991). Furthermore, *Naegleria* and *Acanthamoeba* species have been associated with a wide variety of pathogenic conditions in both man and animals (Martinez, 1977; Dorsch et al., 1983).

Recent results from our laboratory have indicated that alligator serum has antibacterial properties (Merchant et al., 2003). Other studies have suggested that the sera of wetland species, such as bullfrogs, raccoons, muskrats, etc., exhibit strong amoebacidal activity (John and Smith, 1997). Because alligators are largely aquatic organisms, it is not unreasonable to expect that they might exhibit immune responses that would prevent infection and colonization by potentially infectious amoeba species. This study was conducted to investigate the amoebacidal effects of alligator serum.

MATERIALS AND METHODS

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 15 feet long × 6 feet wide. Several small alligators (up to 3 feet in length) were housed in a single tank. Adult alligators were typically captured at night with use of a cable snare.

The environment in the tanks was of 50% dry bottom and 50% water of approximate 6 inches depth. The temperature was maintained at approximately 87–88 F. The alligators were fed formulated dry pellets 4–5 times per week, and the cages were cleaned 5 times per week. Blood samples were drawn from the supravertebral branch of the internal jugular vein using a 1.5-inch 18-gauge needle and a 10-ml syringe (Olsen et al., 1977) and transferred to serum vacutainer tubes.

Amoeba cultures

The following amoebae cultures were obtained from American Type Culture Collection (Manassas, Virginia): *Naegleria gruberi* (30540), *Naegleria australiensis* (30958), *Naegleria lovaniensis* (30569), *Acanthamoeba palestinensis* (50708), *Acanthamoeba polyphaga* (30871), *Acanthamoeba castellanii* (30010), and *Acanthamoeba lenticulata* (50427). *Naegleria* and *Acanthamoeba* species were cultivated axenically using modified PYNFH medium obtained from American Type Culture Collection (Manassas, Virginia). The amoebas were maintained in 5-ml cultures in 7-ml sterile polypropylene tubes at 22 C.

Amoebacidal activity assays

Amoebae cultures were centrifuged in a swinging bucket rotor at 1,500 *g* for 15 min. Approximately 4 ml of medium was removed by vacuum aspiration. The cellular densities of the amoebae cultures were determined using a hemacytometer. The cultures were diluted to the desired density for each experiment using PYNFH medium. The cultures not treated with serum were used as negative controls. The experimentally determined cellular densities of these cultures were designated as 100% survival for each experiment. The cultures treated with alligator serum were expressed as a percentage of the positive control and were thus expressed as percent of maximum amoeba survival.

Temperature dependence of amoebacidal activity

To examine the effects of temperature on the effects of alligator serum on amoeba survival, each culture was incubated with the serum at the various temperatures indicated for 30 min. Controls were included, in which the amoeba cultures were incubated with PYNFH medium and incubated at the same temperatures, to ensure that the temperature shock had no effect on the amoeba survival. For the comparison of the susceptibility of the different species of amoebas, each strain was diluted to 1×10^5 cells/ml and exposed to the different dilutions of alligator serum for 30 min. Survival of each culture was determined by manual counting using a hemacytometer.

Statistics and controls

All experiments were performed in quadruplicate to obtain valid statistical evaluation of the results. All results represent the means ± standard deviation. The results obtained from each experiment were subjected to analysis of variance using Scheffe's post hoc comparisons (Tamhane and Dunlop, 2000).

RESULTS

The concentration-dependent effects of alligator serum on axenic cultures of *N. gruberi* are shown in Figure 1. Incubation of *N. gruberi* with 25% alligator serum for 30 min resulted in 73.7% amoeba survival, whereas 50%, 75%, and undiluted serum produced 46.4%, 31.9%, and 16.7% survival, respectively.

The saturable nature of the amoebacidal effects of alligator serum is shown in Figure 2. Exposure of *N. gruberi*, at a density of 3×10^4 cells/ml, to undiluted alligator serum for 30 min

Received 12 January 2004; revised 22 March 2004; accepted 22 March 2004.

* Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, 5476 Grand Chenier Highway, Grand Chenier, Louisiana 70643.

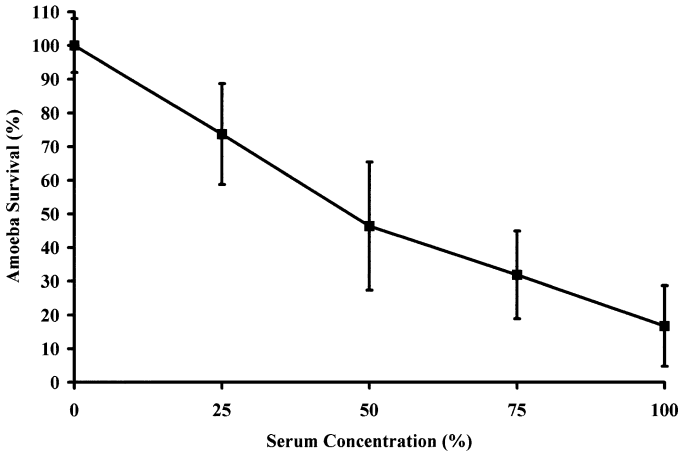

FIGURE 1. Concentration-dependent amoebacidal properties of alligator serum. *Naegleria gruberi* amoeba cultures (10^5 cells/ml) were exposed to alligator serum for 30 min at 22 C. The amoeba cell density of each culture was determined using a hemacytometer under ×40 magnification. The results are expressed as the percent amoeba survival, on the basis of the total amoeba cell density exposed to the serum, and represent the means ± standard deviations for 3 determinations.

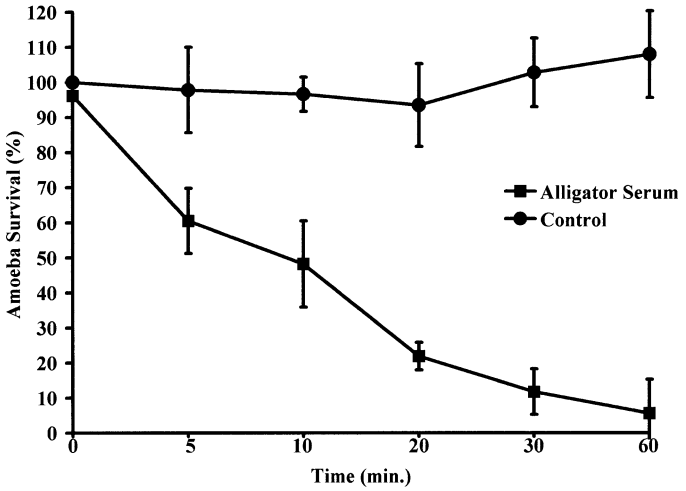

FIGURE 3. The kinetic properties of the amoebacidal effects of alligator serum. Different cellular densities of *Naegleria gruberi* amoeba cultures (10^5 cells/ml) were exposed to undiluted alligator serum for varying periods of time at 22 C. The amoeba cell density of each culture was determined using a hemacytometer under ×40 magnification. The results are expressed as the percent amoeba survival, on the basis of the total amoeba cell density exposed to the serum, and represent the means ± standard deviations for 3 determinations.

resulted in 26 ± 8.2% survival of amoebae. However, incubation of higher densities of amoebae resulted in decreasing effectiveness of the amoebacidal effects of the alligator serum. For instance, incubation of serum with 5×10^4 and 7×10^4 cells/ml resulted in 34.7 ± 6.2% and 40.5 ± 4.7% survival rates, respectively. Exposure of *N. gruberi* densities of 12×10^4, 22×10^4, and 27×10^4 cell/ml to alligator serum resulted in survival rates that were not statistically different from 100% ($P > 0.05$).

The kinetic properties of the amoebacidal activity of alligator serum are shown in Figure 3. Incubation of alligator serum with *N. gruberi*, at a cell density of 8×10^4, resulted in 60.5 ± 9% amoeba survival after only 5 min. The survival rates of the amoeba cultures declined to 48.2 ± 12%, 21.9 ± 4%, 11.8 ± 7%, and 5.6 ± 10% after incubation for 10, 20, 30, and 60 min, respectively. The control cultures, *N. gruberi* incubated with PYNFH culture medium, were not statistically different ($P > 0.05$) from 100% survival at any time point.

The comparative effects of alligator serum on 3 *Naegleria* and 4 *Acanthamoeba* species of amoeba species are shown in Figure 4. All concentrations of serum tested exhibited significant reductions ($P > 0.01$) in amoeba survival for all 7 strains. The alligator serum exhibited concentration-dependent effects

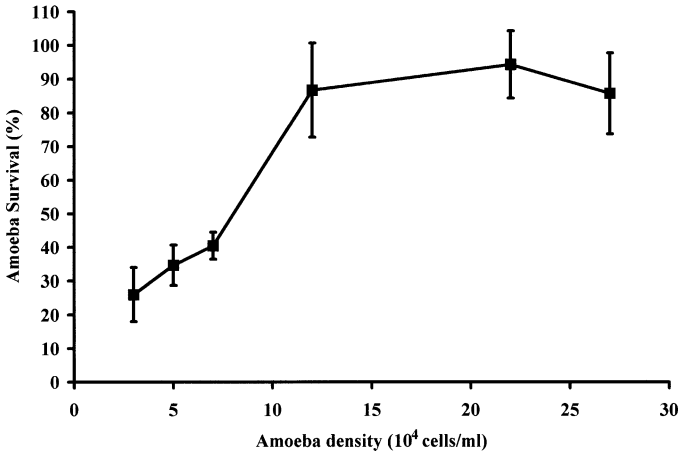

FIGURE 2. The effect of amoeba cell density on the amoebacidal effects of alligator serum. Different cellular densities of *Naegleria gruberi* amoeba cultures were exposed to alligator serum for 30 min at 22 C. The amoeba cell density of each culture was determined using a hemacytometer under ×40 magnification. The results are expressed as the percent amoeba survival, on the basis of the total amoeba cell density exposed to the serum, and represent the means ± standard deviations for 3 determinations.

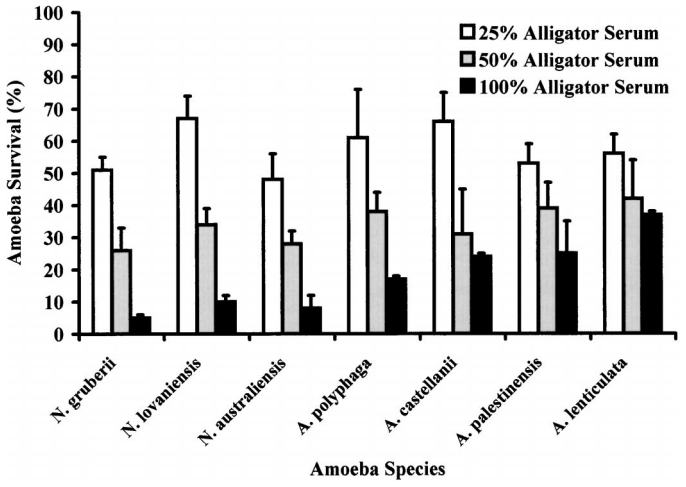

FIGURE 4. The amoebacidal effects of alligator serum on *Naegleria* and *Acanthamoeba* species. Seven different species of amoebae were exposed to alligator serum for 30 min at 22 C. The amoeba cell density of each culture was determined using a hemacytometer under ×40 magnification and adjusted to 10^5 cells/ml. The results are expressed as the percent amoeba survival, on the basis of the total amoeba cell density exposed to the serum, and represent the means ± standard deviations for 3 determinations.

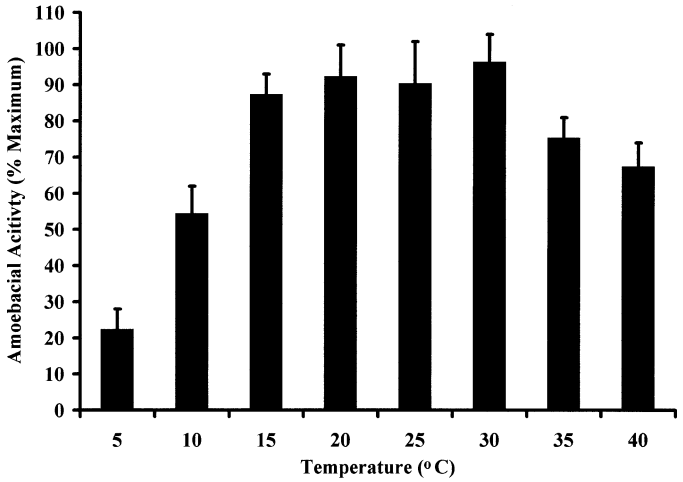

FIGURE 5. The effects of temperature on the amoebacidal effects of alligator serum. Different cellular densities of *Naegleria gruberi* amoeba cultures (5×10^4 cells/ml) were exposed to alligator serum for 30 min at different temperatures. The amoeba cell density of each culture was determined using a hemacytometer under ×40 magnification. The results are expressed as the percent amoeba survival, on the basis of the total amoeba cell density exposed to the serum, and represent the means ± standard deviations for 3 determinations.

on the survival of each amoeba species tested. The effect of 25% serum was most potent for *N. australiensis* (48 ± 8% survival), and exhibited the least effect on *N. lovaniensis* (67 ± 7% survival). The effects of 50% alligator serum were more potent than 25% serum on all species. The largest effect was observed on *N. gruberi* (26 ± 7% survival) and the least effect on *A. lenticulata* (58 ± 12% survival). The effects of 100% alligator serum were the most profound for all 7 species. The 100% serum was most active on *N. gruberi* (5 ± 1% survival) and had the least effect against *A. lenticulata* (37 ± 1% survival).

Preheating serum at 56 C for 30 min resulted in 85 ± 8% amoeba survival. Pretreatment of alligator serum with 20 units of protease, derived from *Streptomyces griseus*, before exposure to the amoebae resulted in 90 ± 6% amoeba survival, relative to amoeba cultures treated with the protease alone. Addition of 30 mM ethylenediaminetetraacetic acid (EDTA) to the serum before exposure to *N. gruberi* resulted in 88 ± 6% survival, relative to amoeba cultures exposed to 30 mM EDTA in the absence of serum.

The temperature dependence of alligator serum–mediated amoebacidal activity is illustrated in Figure 5. Exposure of *N. gruberi* cultures (8×10^4 cells/ml) to alligator serum at temperatures below 15 C resulted in reduced amoebacidal activity. Incubation of serum with amoeba cultures at 5 C resulted in 22 ± 3% of maximal amoebacidal activity, relative to control cultures diluted with medium and incubated at the same temperature, whereas incubation at 10 C produced 54 ± 6% activity. Similarly, incubation at temperatures of 35 and 40 C produced 75 ± 6% and 67 ± 7% of maximal activity, respectively.

DISCUSSION

Amoebae are ubiquitous and have been found associated with a variety of reptilian species. Schuster et al. (2003) described the isolation of *Paravahlkampfia ustiana* amoebae from the gut of moribund pink-tongued skinks (*Hemisphaeriodon gerrardi*). Sesma and Ramos (1989) reported heavy infestations of both *Naegleria* and *Acanthamoeba* species in lizards captured in the Canary Islands. Madrigal and Sesma (1988) found *Naegleria* and *Acanthamoeba* in 3 other species of saurian lizards, whereas Bosch and Deichsel (1972) reported the isolation of both types of amoebae from a wide variety of reptilian species. A variety of amoeba species from different genera have been reported to be pathogenic to reptiles. Kojimoto et al. (2001) have reported the presence of several species of *Entamoeba* species in 4 ball pythons that died from severe hemorrhagic colitis. Species of *Naegleria* and *Acanthamoeba* were found associated with necrotic tissues of the emerald basilisk (Walochnik et al., 1999).

John and Smith (1997) have reported that the serum from some aquatic animals exhibit amoebacidal activity. Their data revealed that animals such as raccoons and bullfrogs exhibit potent amoebacidal activities, whereas more terrestrial animals such as squirrels and rabbits exhibit marginal or no activity. The data presented in Figure 1 illustrate the amoebacidal activity of alligator serum. These data support the hypothesis that some aquatic animals may develop immunity to colonization and infection by potentially infectious amoeba parasites.

Figure 2 illustrates that the capacity of alligator serum to kill *N. gruberi* can be saturated at high amoeba densities. At amoeba densities greater than 12×10^4, the serum complement system of the alligator is apparently overwhelmed and can no longer kill an appreciable percentage of the amoebas. These data show that the same approximate absolute number of amoebae were killed in each culture, but the number killed constitutes an increasingly smaller percentage of the total number of amoeba exposed to the serum as the cell density increases. By multiplying the percentage of amoebae killed by the total number of amoebae in the culture, we derive the total number of amoebae that are killed by 1 ml of alligator serum to be 1.5×10^4–4.0×10^4.

The kinetics of the amoebacidal effects of alligator serum is similar to its antibacterial effects in vitro (Fig. 3; Merchant et al., 2003). The effects are rapid, with amoeba lysis occurring within 5 min of exposure to the serum. Upon exposure, the *N. gruberi* amoebae change shape from the typical flattened, amorphous form to a spherical morphology (data not shown). We propose that this change in shape is experienced to minimize the surface area of the organism that is exposed to the serum. The amoebae that were lysed by alligator serum were observed to quickly dissipate, and thus any amoeba observed under the microscope was judged to be alive.

Alligator serum exhibits broad-spectrum amoebacidal activity (Fig. 4). The capacity to kill was dose dependent for all species of amoebae, with the highest concentration of serum having the greatest impact on amoeba survival. *Acanthamoeba* species have been reported to be completely resistant to serum complement lysis by normal human serum (Toney and Marciano-Cabral, 1998). The data depicted in Figure 4 reveal that alligator serum exhibits more potent amoebacidal effects on the *Naegleria* species than the *Acanthamoeba* species. However, the *Acanthamoeba* species do show appreciable susceptibility, particularly with undiluted alligator serum.

The thermal curve for the effects of alligator serum on the

TABLE I. Undiluted alligator serum was heat treated (56 C, 30 min) or treated with protease for 30 min. The results represent the means ± standard deviations for 4 determinations.

Treatment	Amoeba survival (% maximum)
Alligator serum (untreated)	6 ± 1
Heat-treated alligator serum	85 ± 8
Protease-treated alligator serum	90 ± 6
EDTA-treated alligator serum	88 ± 6

survival of *N. gruberi* is almost identical to that of the antibacterial activity against *Escherichia coli* previously reported (Merchant et al., 2003). The innate immune activity against *N. gruberi* was optimal at 30 C. These data corroborate data collected by other investigators that suggest alligators are biochemically and physiologically optimized at 30–31 C (Seebacher, Guderley et al., 2003). The marked reduction in amoebacidal activity at 5 and 10 C suggests that alligators may be immunocompromised during the winter months, when their core body temperatures can fall to less than 10 C (Brisbin et al., 1982; Seebacher, Elsey et al., 2003). The reduction in activity at temperatures above 35 C may be physiologically irrelevant because wild alligators generally maintain body temperatures below these levels in thermoregulation studies.

The effects of heat, protease, and EDTA treatment on the amoebacidal properties of alligator serum are shown in Table I. Heat treatment of alligator serum (56 C, 30 min.) reduces its amoebacidal activity by 85%. The reduction in the innate immunity by relatively modest heat treatment is characteristic of serum complement activity. In addition, this activity is reduced by 90% by pretreating the alligator serum with protease before exposure to amoeba cultures. This result suggests that the amoebacidal activity is caused by the action of 1, or more, proteins. The amoebacidal activity is also inhibited by 88% in the presence of 30 mM EDTA. Unpublished reports from our laboratory have shown that the alligator serum, similar to human serum (Levine et al., 1953), requires divalent metal ions for activity. Collectively, these data support the role of a serum complement system in the amoebacidal activity of alligator serum. However, it is interesting to note that amoeba survival was not completely reduced by heat, protease or EDTA treatment of the alligator serum. These data indicate that factors, other than serum complement proteins, may be responsible for the amoebacidal activities observed.

The amoebacidal activities reported in this study are comparable with the antibacterial activities reported previously for alligator serum (Merchant et al., 2003). The similarity in kinetic curves, concentration dependence, and temperature-dependent responses lead us to believe that the molecular mechanisms involved are the same for both the antibacterial and amoebacidal effects. Furthermore, the thermal instability, protease susceptibility, and sensitivity to EDTA suggest that the amoebacidal effects of alligator serum are, in part, due to an active serum complement system. Further studies in our laboratory have indicated the presence of complement activity (M. Merchant, D. Thibodeaux, K. Loubser, and R. M. Elsey, pers. obs.). To our knowledge, this is the first report of amoebacidal activity in any crocodilian species.

ACKNOWLEDGMENTS

The authors wish to thank Phillip ''Scooter'' Trosclair and Dwayne LeJeune, of the Louisiana Department of Wildlife and Fisheries, for their assistance with catching and handling wild alligators.

LITERATURE CITED

BOSCH, I., AND G. DEICHSEL. 1972. Morphological study of pathogenic and potentially pathogenic amoebae of the types *Entamoeba* and *Hartmannella-Acanthamoeba* from reptiles. Zentralblatt feur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene **40:** 107–129.

BRISBIN, I. L. JR., E. A. Standora, and M. J. Vargo. 1982. Body temperatures and behavior of American alligators during cold winter weather. American Midland Naturalist **107:** 209–218.

DORSCH, M. M., A. S. CAMERON, AND B. B. ROBINSON. 1983. The epidemiology and control of primary amoebic meningoencephalitis with particular reference to South Australia. Transactions of the Royal Society Tropical Medicine Hygiene **77:** 372–377.

JOHN, D. T., AND B. L. SMITH. 1997. Amebacidal activity of wild animal serum. Journal of Parasitology **83:** 757–759.

KOJIMOTO, A., K. UCHIDA, Y. HORII, S. OKUMURA, R. YAMAGUCH, AND S. TATEYAMA. 2001. Amebiasis in four ball pythons, *Python reginus*. Journal of Veterinary Medical Science **63:** 1365–1368.

LEVINE, L., A. G. OSLER, AND M. M. MAYER. 1953. The role of calcium and magnesium ions in complement fixation and immune hemolysis. III. The respective roles of calcium and magnesium ions in immune hemolysis. Journal of Immunology **71:** 374–379.

MADRIGAL, C., AND M. J. SESMA. 1988. Isolation of free-living amoebae, potentially pathogenic for humans, from 3 species of saurians from the western Canary Islands. Revista de Sanidad E Higiene Publica (Madrid) **62:** 1405–1409.

MARCIANO-CABRAL, F. 1988. Biology of *Naegleria* spp. Microbiological Reviews **52:** 114–133.

———, AND G. CABRAL. 2003. *Acanthamoeba* spp. As agents of disease in humans. Clinical Microbiological Reviews **16:** 273–303.

MARTINEZ, A. J. 1977. Free-living amebic meningoencephalitides: Comparative study. Neurologia, Neurocirugia, Psiquiatria **18:** 391–401.

———, AND K. JANITSCHKE. 1985. *Acanthamoeba*, an opportunistic microorganism: A review. Infection **13:** 251–256.

———, AND G. S. VISVESVARA. 1991. Laboratory diagnosis of pathogenic free-living amoebas: *Naegleria*, *Acanthamoeba*, and *Leptomyxid*. Clinical Laboratory Medicine **11:** 861–872.

MERCHANT, M., C. ROCHE, R. M. ELSEY, AND J. PRUDHOMME. 2003. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). Comparative Biochemistry and Physiology B. **136:** 505–513.

OLSEN, G., J. HESSLER, AND R. FAITH. 1977. Technics for the blood collection and intravascular infusion of reptiles. Laboratory Animal Science **25:** 783–786.

SCAGLIA, M. 1997. Human pathology caused by free-living amoebae. Annali Dell Istituto Superiore di Sanita (Roma) **33:** 551–566. [In Italian.]

SCHUSTER, F. L., J. F. DE JONCKHEERE, H. MOURA, R. SRIRAM, M. M. GARNER, AND G. S. VISVESVARA. 2003. Isolation of a thermotolerant *Paravahlkampfia* sp. from lizard intestine: Biology and molecular identification. Journal of Eukaryotic Microbiology **50:** 373–378.

SEEBACHER, F., R. M. ELSEY, AND P. L. TROSCLAIR III. 2003. Body temperature null distributions in reptiles with nonzero heat capacity: Seasonal thermoregulation in the American alligator (*Alligator mississippiensis*). Physiology and Biochemical Zoology **76:** 348–359.

———, H. GUDERLEY, R. M. ELSEY, AND P. L. TROSCLAIR III. 2003. Seasonal acclimatisation of muscle metabolic enzymes in a reptile (*Alligator mississippiensis*). Journal of Experimental Biology **206:** 1193–1200.

SESMA, M. J., AND L. Z. RAMOS. 1989. Isolation of free-living amoebas from the intestinal contents of reptiles. Journal of Parasitology **75:** 322–324.

TAMHANE, A. C., AND D. DUNLOP. 2000. Scheffe' method for general contrasts. *In* Statistics and data analysis: From elementary to intermediate, 1st ed. Prentice Hall, Upper Saddle River, New Jersey, p. 477–477.

TONEY, D. M., AND F. MARCIANO-CABRAL. 1998. Resistance of *Acanthamoeba* species to complement lysis. Journal of Parasitology **84:** 338–344.

WALOCHNIK, J., A. HASSL, K. SIMON, G. BENYR, AND H. ASPOCK. 1997. Isolation and identification by partial sequencing of the 18S ribosomal gene of free-living amoebae from necrotic tissue of *Basilliscus plumifrons* (Sauria: Iguanidae). Parasitology Research **85:** 601–603.