PATHOLOGY OF EXPERIMENTAL MYCOPLASMOSIS IN AMERICAN ALLIGATORS

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ABSTRACT: Mycoplasma alligatoris was the suspected etiology of an epidemic of acute multisystemic inflammatory disease which emerged in captive American alligators (Alligator mississippiensis) in Florida (USA) in 1995. In an experimental inoculation study conducted from April through October 1999, 18 alligators were inoculated with 102, 104, or 106 colony forming units (CFU) of M. alligatoris by instillation into the glottis. As early as 1 wk post-inoculation (PI), mycoplasma were cultured from blood of three of six alligators inoculated with 106 CFU. Two of those died and the third was euthanatized within 4 wk PI. Necropsy gross findings included fibrinous polyserositis and polyarthritis. Histopathologic changes in affected individuals included pulmonary edema, interstitial pneumonia, pericarditis, myocarditis, meningoencephalomyelitis, and synovitis. Mycoplasma were cultured quantitatively in high numbers from trachea, lung, coelomic cavity, liver, spleen, interior of pericardial sac, heart, blood, brain, and limb joints. In alligators inoculated with 106 CFU, heterophilia and moderate hyperglycemia peaked about 4 wk PI, and seroconversion occurred by 6 to 8 wk PI. Necropsy gross and histologic findings were generally unremarkable for the surviving alligators inoculated with 106 CFU, alligators inoculated with 102 or 104 CFU, and four uninoculated control alligators. Mycoplasma were not cultured at any time point from those alligators. The findings confirm that M. alligatoris can cause fulminant inflammatory disease and rapid death of alligators.

Key words: Alligator mississippiensis, Mycoplasma alligatoris, American alligator, mycoplasmosis, pathology.

INTRODUCTION

An epidemic of acute fatal multisystemic inflammatory disease, including pneumonia, pericarditis, and multifocal arthritis, emerged in captive American alligators (Alligator mississippiensis) in St. Johns County, Florida, USA, in 1995. The affected group was composed of 200 to 300 kg adult males that were older than 30 yr, housed in a 20 x 50 m outdoor sandy enclosure with a central spring-fed pool. Thirty-three alligators died and thirteen moribund alligators were euthanatized within 1 mo of the index case. No probative abnormalities in blood cell counts or plasma biochemistry were observed. Results of toxicant, heavy metal, mineral, and vitamin analyses of tissues and serum were unremarkable. Results of routine aerobic and anaerobic bacterial culture of blood and lung tissue samples were uninformative regarding the etiology of the disease. Attempts to isolate virus from pneumatic lung tissue samples were unsuccessful. However, a previously undescribed species of mycoplasma, Mycoplasma alligatoris (Brown et al., 2001a), was later cultured from multiple tissues and body fluids of affected alligators. No mycoplasma could be cultured or detected by polymerase chain reaction (PCR) from blood or tissues of five healthy control alligators that were euthanatized for diagnostic necropsy, which finding implicated M. alligatoris as a possible etiologic agent (Clippinger et al., 2001).

A subsequent pilot experimental inoculation study fulfilled the Henle-Koch-Evans postulates for M. alligatoris as the etiology of fatal mycoplasmosis of alligators. Four healthy alligators were inoculat-
ed with $1 \times 10^6$ colony forming units (CFU) of *M. alligatoris* by intracoelomic injection ($n = 2$) or by instillation into the glottis ($n = 2$). A control alligator received sterile broth. Three of four inoculated alligators died between 1 and 3 wk post-inoculation (PI) with systemic mycoplasmosis. Regardless of inoculation route, *M. alligatoris* was re-isolated post-mortem from multiple organs and body fluids. Pathology included diffuse interstitial pneumonia, severe fibrinous pericarditis, arthritis of the stifle and elbow joints, lymphocytic periportal hepatitis, lymphocytic interstitial nephritis, and lymphoid splenic hyperplasia (Clippinger et al., 2001). The 6-wk PI anti-*M. alligatoris* antibody titer of the surviving alligator rose to 1:640 and remained constant for the following 8 wk. The control remained seronegative (titer 1:<10) and free of mycoplasma at necropsy after 14 wk (Brown et al., 2001a). The findings were remarkable because mycoplasmosis generally occurs as a chronic disease which, with the exception of *Mycoplasma mycoides* subsp. *mycoides* in ruminants, is rarely lethal in animals.

Understanding the pathogenesis of *M. alligatoris* mycoplasmosis is essential to prevent or respond to possible future epidemics. In this paper we report the results of detailed pathological, hematological, and bacteriological examinations of alligators following experimental inoculation with different doses of *M. alligatoris*.

**MATERIALS AND METHODS**

Twenty-two young adult female American alligators (116 to 147 cm; 6 to 13 kg) were obtained from the St. Augustine Alligator Farm and Zoological Park (St. Augustine, Florida, USA) in April, 1999. The alligators were acclimated for 6 wk post-intake. Alligators ($n = 6$ per group) were inoculated by instillation into the glottis of 1 ml of SP4 broth containing $1 \times 10^2$, $1 \times 10^4$, or $1 \times 10^6$ CFU of third-passage *M. alligatoris* strain A21JP2\(^T\). As negative controls, four alligators received either inoculation with sterile broth or no treatment of any kind. Blood samples were obtained from the supravertebral sinus in lithium-heparinized glass containers from all alligators at weekly intervals for 4 wk, then biweekly, until the alligators died or were euthanatized 12 to 16 wk PI. For mycoplasma culture at each time point, 20 μl of blood was added to 1 ml of SP4 broth, then 20 μl of that culture was inoculated onto SP4 agar. Broths and agar plates were incubated as described above. Mycoplasma growth in broth was detected by a color change from red to yellow as a result of acidification of the broth following glucose fermentation. Mycoplasma colonies

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growing on agar plates were visualized by using a dissecting microscope. Plasma was stored at 
−70 C in polypropylene cryovials for ELISA. Because of clinical laboratory constraints, only 
a subset of the blood samples could be analyzed by hematology and clinical biochemistry 
at each time point.

A complete diagnostic necropsy was performed on each alligator. Necropsies were 
completed by October 1999. For euthanasia, 0.5 ml/kg of a solution containing 390 mg/ml of 
pentobarbital sodium and 50 mg/ml phenytoin sodium (Benthana- 

results were also stained with Perls iron stain (Luna, 1968). Tissue sections were examined by using light 

RESULTS

Mycoplasma alligatoris was cultured as early as 1 wk PI from peripheral blood of 
three alligators inoculated with 10^6 CFU. One of those (accession number AA168067) 
died abruptly after 1 wk PI, another (AA165094) died abruptly after 4 wk PI, 
and the third (AA165089) was euthanized after 4 wk PI because it had sudden onset of overt 
signs of neuromuscular dysfunction including gaping mouth and postural instability. Disseminated mycoplas-
mosis was demonstrated at necropsy of those alligators by positive cultures obtained from every sample except 
ears and conjunctivae (Table 1). Mycoplasma were not cultured at any time point from peripheral 

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Samples of tissues (trachea, lung, pericardi-

Hematological and plasma biochemical data were incomplete enough that meaningful statistical analyses of effects of inoculation with M. alligatoris were not possible. In alligators inoculated with 10^6 

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to 4 wk PI, and blood glucose was elevated about 2-fold from baseline by 3 to 4 wk PI, consistent with systemic inflammatory disease. Moderate lymphocytopenia (about a 50% decrease from baseline in the number of lymphocytes) was observed at necropsy of the alligators that died. No other effects on other blood cell counts, plasma electrolytes, or liver function indicators in any group were noted.

Statistical power to detect seroconversion in the group inoculated with 10⁶ CFU was reduced because half of those alligators died before specific antibody could be produced (Brown et al., 2001b). The mean anti-\textit{M. alligatoris} antibody level among the surviving alligators in that group rose to about 2-fold higher \((P < 0.05)\) than the pre-inoculation mean beginning 6 wk PI. Trends toward seroconversion in the groups inoculated with 10² or 10⁴ CFU were not significant. Uninoculated controls did not seroconvert.

Heart blood samples cultured for bacteria other than mycoplasma were negative at necropsy except for alligators that had superficial skin lesions or bite wounds. \textit{Aeromonas hydrophila}, \textit{Micrococcus kristinae}, \textit{Proteus vulgaris}, and \textit{Vibrio parahemolyticus} were cultured from blood of one alligator in the uninoculated control group, which had a bite wound on its leg. \textit{Staphylococcus cohnii} was cultured from blood of one alligator in the low-dose group, which had a bite wound on its leg. \textit{Sebekia mississippiensis} was recovered from the lungs, and a single male ascarid \textit{Dujardinascaris waltoni} was present in the stomach, at necropsy of another alligator in the high-dose group.

Gross anatomical and histological findings at necropsy were normal for all alligators except for another alligator in the high-dose group. This alligator had a bite wound on its leg, had bacteremia of \textit{A. hydrophila}, \textit{P. vulgaris}, and \textit{V. parahemolyticus}.

### Table 1. Association of \textit{Mycoplasma alligatoris} infection with histological lesions in alligators.

<table>
<thead>
<tr>
<th>Site</th>
<th>Lesion scores</th>
<th>Lesion scores</th>
<th>Lesion scores</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>\textit{Mycoplasma}</td>
<td>\textit{Mycoplasma}</td>
<td>\textit{Mycoplasma}</td>
</tr>
<tr>
<td>Trachea(^{d})</td>
<td>&lt;30 mild</td>
<td>&lt;30 normal</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Lung(^{e})</td>
<td>7.4 x 10⁹ mild</td>
<td>1.0 x 10⁴ normal</td>
<td>4.9 x 10¹ mild</td>
</tr>
<tr>
<td>Pericardium(^{d})</td>
<td>8.9 x 10⁹ severe</td>
<td>4.2 x 10⁵ mild</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Heart(^{f})</td>
<td>&gt;3 x 10¹⁰ severe</td>
<td>9.1 x 10³ mild</td>
<td>&lt;30 mild</td>
</tr>
<tr>
<td>Spleen(^{e})</td>
<td>4.7 x 10⁹ normal</td>
<td>3.7 x 10³ normal</td>
<td>1.0 x 10² mild</td>
</tr>
<tr>
<td>Liver(^{e})</td>
<td>8.6 x 10⁹ severe</td>
<td>1.1 x 10⁴ normal</td>
<td>1.0 x 10² normal</td>
</tr>
<tr>
<td>Brain(^{f})</td>
<td>1.2 x 10⁶ severe</td>
<td>1.1 x 10³ mild</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Left elbow(^{d})</td>
<td>3.9 x 10³ normal</td>
<td>&lt;30 normal</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Right elbow(^{d})</td>
<td>3.7 x 10³ normal</td>
<td>&lt;30 normal</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Left knee(^{d})</td>
<td>5.3 x 10² normal</td>
<td>8.3 x 10⁸ moderate</td>
<td>negative normal</td>
</tr>
<tr>
<td>Right knee(^{d})</td>
<td>8.0 x 10¹ normal</td>
<td>4.6 x 10⁸ moderate</td>
<td>negative normal</td>
</tr>
<tr>
<td>Tonsils(^{f})</td>
<td>negative mild</td>
<td>negative normal</td>
<td>&lt;30 normal</td>
</tr>
<tr>
<td>Coelomic cavity(^{d})</td>
<td>6.2 x 10⁷ N/A(^{f})</td>
<td>1.3 x 10⁵ N/A(^{f})</td>
<td>negative N/A(^{f})</td>
</tr>
</tbody>
</table>

\(^{a}\) Animal accession number.
\(^{b}\) Colony forming units per sample.
\(^{c}\) Normal was occasional heterophil or lymphocyte, and no edema or changes in tissue structure. Mild changes were small multifocal lymphoid aggregates, small numbers of diffuse heterophils or lymphocytes, mild edema, and mild changes in tissue structure. Moderate changes were multifocal to focally extensive lymphoid aggregates, moderate numbers of diffuse heterophils and lymphocytes, moderate edema, and disorganization of tissue structure. Severe changes were large numbers of heterophil and lymphocyte infiltrates, marked edema, and necrosis.

AA165089\(^{a}\) Mycoplasma\(^{b}\) Lesion scores\(^{c}\) AA165084\(^{a}\) Mycoplasma\(^{b}\) Lesion scores\(^{c}\) AA165076\(^{a}\) Mycoplasma\(^{b}\) Lesion scores\(^{c}\)

AA168067\(^{a}\) Mycoplasma\(^{b}\) Lesion scores\(^{c}\)

\(^{d}\) Sample was 0.5 g minced tissue.
\(^{e}\) Sample was an ultrafine-tip nylon swab.
\(^{f}\) Not assessed.
gators in the uninoculated control (including the individual with Gram-negative bacteremia), lowest-dose, and medium-dose groups, and for the three alligators in the highest-dose group which did not develop *M. alligatoris* septicemia. Among the three alligators that died with disseminated mycoplasmosis, gross pathologic findings at necropsy (Table 1) were variable but presented an overall picture of acute multisystemic inflammatory disease. In affected alligators the lungs had local purple discoloration with congestion and fibrinous pleuritis (Fig. 1). Edema fluid, heterophils, and small numbers of mononuclear cells were present in the lung interstitium. The thickened pericardium showed moderate fibrinous pericarditis to pyogranulomatous inflammation (Fig. 2). The myocardium displayed small multifocal areas of necrosis to severe fibrinous pyogranulomatous myocarditis, with vacuoles within the myocardiun, and endocarditis. Fibrin and serous exudate were present within the thickened liver capsule (Fig. 3), and the surface of the liver had pyogranulomatous inflammation. Small numbers of mononuclear cells and increased numbers of lymphocytes were present in periportal areas. The spleen displayed loss of normal architecture, multifocal areas of necrosis, and lymphoid follicle depletion, with increased numbers of mononuclear cells, macrophages, and heterophils throughout the spleen. The forebrain, midbrain, hindbrain, and cerebellum displayed diffuse moderate to severe heterophilic lymphocytic or pyogranulomatous meningitis (Figs. 4, 5). No significant gross or histological changes were noted in the kidneys. Affected alligators had arthritis of one or more limb joints, with subcutaneous edema and cloudy yellow inspissated synovial fluid. There was mild to moderate thick-
FIGURE 3. Fibrin sheets within thickened liver capsule following inoculation of an American alligator with $1 \times 10^6$ CFU of Mycoplasma alligatoris. Bar = 2 cm.

FIGURE 4. Histologic section of brain of an American alligator showing normal meninges following sham inoculation with sterile broth. Bar = 200 μm.

DISCUSSION

The disease following experimental inoculation of alligators with *M. alligatoris* was clinically similar to that observed during the natural epidemic which occurred in 1995 among captive alligators in Florida. The disease was acute, with death occurring as early as 1 wk after exposure. Indicators of the disease were nonspecific reflections of multisystemic inflammation, such as lethargy and edema, and moderate hematologic and plasma biochemical changes. The pathology following experimental inoculation of alligators with $1 \times 10^6$ CFU of *M. alligatoris* was also similar to that resulting from the natural disease. Pathologic changes associated with the mycoplasma infection were fibrinous polyserositis and polyarthritis, leading to fulminant inflammatory disease and death, probably caused by meningitis and pneumonia, in half of the alligators exposed to that dose. Doses of $1 \times 10^2$ or $1 \times 10^4$ CFU of *M. alligatoris* did not cause mycoplasmosis in alligators when instilled into the glottis.

The disease is potentially significant to both captive and wild alligators. Ranches in the U.S. Gulf Coast states annually process approximately 175,000 alligators for hides and meat (Louisiana Cooperative Extension Service, 2000; Lane and King, 1996). Live alligators are frequently exchanged among collections, and are also
exported from the U.S. for commercial production. Once the mycoplasma enters one production facility, there may be significant potential for spread of *M. alligatoris* among ranches with associated economic risk. Alligator ranching is based on collection of eggs and hatchlings from the wild. For that reason, some wild populations are intermittently replenished with juvenile alligators repatriated after “head-starting” hatchlings from eggs collected in the wild. That practice could provide a vector for *M. alligatoris* transmission from captive alligators of diverse origins to local wild populations. Potential spread of mycoplasmosis might now be prevented by appropriate screening by mycoplasma culture or serology.

Because seroconversion in reptiles usually takes 6 to 8 wk (Lerch et al., 1967; Schumacher et al., 1993), an acutely lethal pathogen such as *M. alligatoris* may escape serological detection at necropsy of alligators that die abruptly. Testing of paired plasma samples, obtained at least 8 wk apart, would be necessary to minimize the risk of false negative ELISA results. Any ELISA result should be interpreted in the context of age, sex, and seasonal effects on reptile immune competence (Zapata et al., 1992), which may also influence susceptibility to and severity of infectious diseases. However, *M. alligatoris* should be easily culturable on SP4 medium from blood or minced tissue samples of infected alligators that die abruptly. Alligators which were exposed to $10^6$ CFU of *M. alligatoris* but did not develop clinical disease had seroconverted and seemed to have cleared the mycoplasma infection by 12 wk after exposure, suggesting that seropositive sta-
tus indicated past exposure but not necessarily current infection.

Although A. hydrophila and Gram-negative enteric bacteria have been implicated in septicemia of crocodilians (Novak and Seigel, 1986), A. hydrophila has also been cultured with high frequency from internal tissues of healthy alligators (Gorden et al., 1979). There was no evidence that secondary infections were responsible for the pathologic effects observed in the present study, and M. alligatoris may be retrospectively considered as a possible etiology of alligator deaths without apparent cause formerly attributed to A. hydrophila infection (Gorden et al., 1979). Sebekia mississippiensis and D. waltzoni are also normally found in healthy alligators (Hazan et al., 1978).

One other mycoplasmal disease of crocodilians has been described. Polyarthritis in young Nile crocodiles (Crocodylus niloticus) ranched in Zimbabwe was caused by Mycoplasma crocodyli (Mohan et al., 1995; Kirchhoff et al., 1997). Although M. crocodyli and M. alligatoris are very closely related, as indicated by 98% 16S rRNA gene nucleotide sequence similarity (D. R. Brown et al., unpubl. data), even intracelomic or intrapleural inoculation with 10⁶ CFU of M. crocodyli into Nile crocodiles was not fatal (Mohan et al., 1997). In an experimental inoculation host-range study, conducted concurrently with the present study described above, broad-nosed caimans (Caiman latirostris), which are phylogenetically close relatives of alligators, were similarly susceptible to lethal M. alligatoris infection, while phylogenetically distant Siamese crocodiles (Crocodylus siamensis) became colonized by the mycoplasma but did not experience disease (G. W. Pye, unpubl. data). That finding may have relevance to the yet unidentified reservoir of the 1995 epidemic, which occurred in a collection that included all 23 crocodilian species.

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