

Molecular and antigenic characterization of *Bordetella bronchiseptica* isolated from a wild southern sea otter (*Enhydra lutris nereis*) with severe suppurative bronchopneumonia

Clare M. Staveley¹, Karen B. Register, Melissa A. Miller, Susan L. Brockmeier, David A. Jessup, Spencer Jang

Abstract. *Bordetella bronchiseptica* was isolated in pure culture from the lung, abdomen, and intestine of a wild free-ranging southern sea otter (*Enhydra lutris nereis*) with severe, suppurative bronchopneumonia. Immunohistochemistry, using antiserum raised to *B. bronchiseptica*, revealed strong positive staining of bacteria attached to bronchial ciliated epithelia as well as scattered positive staining in affected alveoli. Western blot analysis demonstrated that virulence factors, filamentous hemagglutinin, pertactin, and adenylate cyclase toxin are produced by the sea otter *B. bronchiseptica* isolate. Ribotype analysis using *Pvu* II restriction digests indicated that this isolate is most similar to strains commonly obtained in domestic dogs and cats.

Bordetella bronchiseptica is a well-documented respiratory tract pathogen affecting a broad range of animal species. It is an aerobic, gram-negative coccobacillus that establishes itself in the ciliated respiratory epithelium of the host. Pathogenic strains have been associated with atrophic rhinitis in pigs and rats and severe bronchopneumonia in dogs, cats, and rabbits. In dogs, *B. bronchiseptica* is considered to be an important pathogen in infectious tracheobronchitis (kennel cough) and frequently contributes to the pneumonia observed in canine distemper.

Bordetella bronchiseptica has been well studied in a wide array of mammalian species, including humans. Only recently were isolates from sea mammals studied. *Bordetella bronchiseptica* was implicated as a key secondary pathogen in conjunction with a phocine morbillivirus epidemic in the North Sea around Great Britain and Denmark in 1988.^{2,6,10} A previous study,¹⁵ in which isolates obtained from seals affected during this outbreak were evaluated by *Pvu* II ribotyping, revealed a single ribotype unique to the seal population.

Bordetella bronchiseptica has not been reported previously from sea otters. This study describes the isolation of *B. bronchiseptica* from a southern sea otter (*Enhydra lutris nereis*) and the histopathological changes associated with this infection. Additionally, the genetic and phenotypic characteristics of the sea otter isolate were examined.

In November 1999, a wild, adult female southern sea otter was found dead on the shore near Carmel Point in Monterey County, California. Severe facial

trauma was noted at the time of collection. Mature male sea otters will often clasp the planum nasale of females during mating, frequently causing soft tissue lacerations.⁵ In this case, the facial trauma was initially attributed to mating interactions. The otter was transported to the California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center in Santa Cruz, California, for postmortem examination.

A detailed gross postmortem examination was performed. Postmortem radiographs were examined for evidence of gunshot, bone fractures, or embedded shark teeth. A variety of tissues, including brain, lung, heart, spleen, multiple lymph nodes, kidney, liver, intestine, urinary bladder, esophagus, trachea, thyroid gland, and adrenal gland were immersion fixed in 10% neutral-buffered formalin (NBF). Trimmed, formalin-fixed tissues were paraffin embedded, and 5 μ m sections were cut, stained with hematoxylin and eosin (HE), and examined by light microscopy.

An alkaline phosphatase system^a was used in tissue sections to detect *B. bronchiseptica* antigens, using polyclonal rabbit anti-*B. bronchiseptica*, as described previously.¹ Sections from a sea otter that was culture-negative for *B. bronchiseptica* and sections lacking primary antibody were used as negative controls. Tissues evaluated using immunohistochemistry included tongue, soft palate/nasopharynx, myocardium, lung, kidney, liver, spleen, thyroid gland, adrenal gland, pancreas, mesenteric and mediastinal lymph nodes, thymus, submandibular salivary gland, bladder, ovary, uterus, small intestine, and stomach. Matched tissue sections were stained with HE.

Sheep blood (BAP) and MacConkey (MAC) agar plates^b were inoculated with swabs obtained from samples of lung, abdominal fluid, and intestinal fluid from the sea otter. All plates were incubated at 37 C in 5%

From the California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, Santa Cruz, CA 95060 (Staveley, Miller, Jessup), USDA/Agricultural Research Service/National Animal Disease Center, Ames, IA 50010 (Register, Brockmeier), and University of California Davis School of Veterinary Medicine, Davis, CA 95616 (Jang). ¹ Corresponding authors.

CO₂ for 48 hours. Bacterial colonies were subcultured onto additional BAP or MAC plates, and isolate identity was confirmed by standard biochemical methods and by cellular fatty acid analysis.^c

Bordetella bronchiseptica strain KM22, a previously described virulent swine isolate,¹ was included in some assays for comparative purposes. Sea otter *B. bronchiseptica* isolates were grown on Bordet-Gengou agar^d for 36 hours at 37 C.

Hemagglutination (HA) studies were carried out in 96-well, V-bottom plates.^e Bacterial suspensions containing 5×10^9 CFU/ml (CFU refers to colony forming units) were serially diluted in phosphate-buffered saline (PBS). An equal volume of bovine, porcine, equine, or ovine red blood cells (RBCs), washed 3 times in PBS and resuspended at a concentration of 1%, was mixed with the bacterial suspension in each well. Negative controls containing RBCs in PBS were included on each plate. Plates were incubated at room temperature, undisturbed, for 2 hours and then scored as follows: 3+, complete agglutination with little or no cell pellet; 2+, moderate to large aggregates but with a cell pellet clearly visible; 1+, small aggregates but with a cell pellet similar to the negative control; and 0, no aggregates and a cell pellet indistinguishable from the negative control. The HA titer was recorded as the reciprocal of the highest dilution giving a 2+ reaction.

Genomic DNA was isolated using a commercially available kit.^f Ribotyping analysis was based on hybridization of *Pvu* II digestion fragments with a portion of the *Escherichia coli* rRNA operon *rmB* followed by chemiluminescent detection as reported.^{13,14}

Bordetella bronchiseptica whole cell extracts or extracts enriched in outer membrane proteins were prepared and processed as described.¹² Extract proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels according to previously described methods⁴ and stained with Coomassie Blue G-250 or electrophoretically transferred to nitrocellulose. Membranes were incubated with monoclonal antibodies specific for filamentous hemagglutinin (X3C),⁸ pertactin (BPE3),³ or adenylate cyclase toxin (ACT)⁷ as described¹² or with undiluted sea otter serum. Binding of monoclonal antibodies was detected using alkaline phosphatase conjugates.¹² Because no anti-sea otter immunoglobulin reagents are commercially available, Protein A and Protein G horseradish peroxidase conjugates were evaluated on dot blots for their ability to bind sea otter antibodies. No binding was detectable using Protein G; however, Protein A was observed to bind at a level similar to that found for rabbit immunoglobulins (data not shown) and was used for blots in which the primary antibody source was sea otter serum.

No bone fractures or other abnormalities were detected on postmortem radiographs. Significant gross abnormalities consisted of emaciation and a large skin and an acute soft tissue laceration on the rostral maxilla with irregular, friable tissue margins. The skin overlying the rostral maxilla, including the entire planum nasale, was missing, and the nasal cavity and nasal cartilage were exposed. A large blood clot was present in the caudal nasopharynx, completely filling this space. Smaller skin and soft tissue lacerations were present in the right and left maxillary gingival margins and caudal maxilla, and a 0.5-cm-long superficial laceration was present on the left sclera near the medial canthus. The gross appearance of these lacerations was consistent with bite wounds. The lungs were pink-red mottled with a moderately firm consistency. The right caudodorsal lung field was gray and firm, and the pleural surfaces were irregularly roughened with a dull appearance, consistent with fibrin deposits. Multifocal petechia and ecchymoses were present on the surfaces of the lungs, epicardium, and peritoneum. Sections of lung floated when placed in 10% NBF, suggesting there was not a significant amount of consolidation.

All sampled tissues were examined by light microscopy. Most sections of lung contained multifocal to coalescing areas of necrotizing and suppurative bronchopneumonia, characterized by large areas of alveolar luminal filling (or obliteration) by neutrophils, large foamy macrophages, cellular debris, and fibrin. Irregular clusters of short, gram-negative bacterial rods were interspersed throughout this exudate within the affected alveolar spaces. Adjacent bronchioles contained similar inflammatory exudates and cellular debris and bacteria (Fig. 1A). Pulmonary interlobular septae were markedly expanded by fibrin and the inflammatory cells. The remaining tissues were histologically unremarkable.

Large numbers of *B. bronchiseptica* were isolated in pure culture from lung, abdominal fluid, and intestine. The isolate, designated SO3287-99, was hemolytic and had colony morphology consistent with a Bvg⁺ phenotype (small, dome-shaped colonies).

Bordetella bronchiseptica antigen was detected by immunohistochemical staining only in the lung. Positive staining was observed at the surface of the ciliated epithelia and within affected alveolar spaces (Fig. 1B). No *B. bronchiseptica* antigen was detected in tissue sections from the negative controls.

On the basis of a comparison with the 19 previously described *B. bronchiseptica* ribotypes,¹³⁻¹⁵ isolate SO3287-99 was determined to be ribotype 4 (Fig. 2, lane 2). Two hundred forty-nine additional *B. bronchiseptica* isolates, acquired from 12 host species, have so far been characterized by *Pvu* II ribotyping.¹³⁻¹⁵ These include 29 ribotype-4 isolates, with 69% ob-

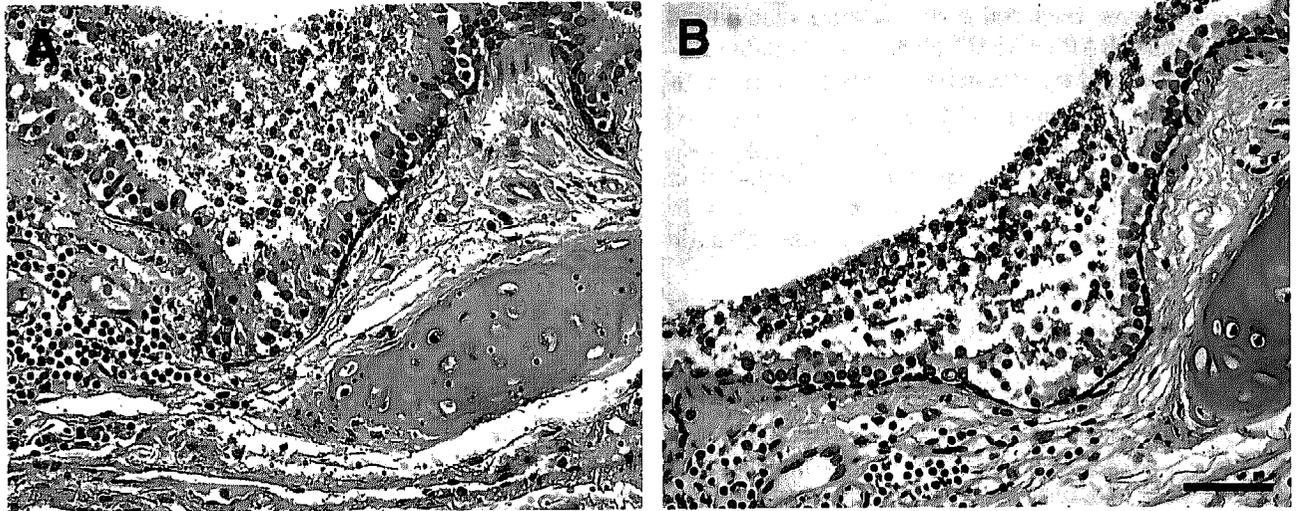


Figure 1. A, HE-stained bronchus from SO3287-99, showing luminal exudates and cellular debris. Scattered exocytosing inflammatory cells, and swollen, necrotic bronchial epithelial cells can be seen within the ciliated epithelium. B, IHC-stain of affected bronchus at the same magnification as in A, showing positive (red) staining of coccobacilli attached to the surfaces of ciliated cells (Bar = 50 μ m).

tained from dogs, 21% obtained from cats, and 10% from horses.

SO3287-99 did not agglutinate equine or ovine RBCs but did agglutinate RBCs of bovine and porcine origin (Table 1). These results are similar to those of KM22, a virulent swine isolate included for comparison, except that titers of SO3287-99 were slightly lower. This result indicates SO3287-99 is a virulent, or Bvg⁺ phase, isolate capable of inducing respiratory disease in susceptible hosts.

In a comparison of whole cell protein profiles, pro-

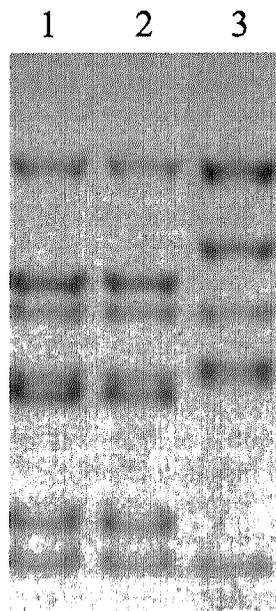


Figure 2. Ribotype analysis of sea otter isolate. Lane 1 = SO3287-99 isolate (sea otter), lane 2 = ribotype 4 (standard), and lane 3 = ribotype 19 (seal isolate).

teins of approximately 89 and 30 kD are apparent in SO3287-99, which are absent or found in lower quantities in KM22. No other differences were noted.

Immunoblotting demonstrated that the virulence factors Filamentous hemagglutinin (FHA) (220 kD), pertactin (68 kD), and ACT (210 kD) are produced by SO3287-99 (Fig. 3). Faster-migrating bands detectable on the FHA blot represent breakdown products of *B. bronchiseptica* and have been described by other investigators⁸ (Fig. 3, panel B). One of the major cleavage products associated with SO3287-99 appears to have a different mobility than the analogous protein in KM22, suggesting there may be some structural heterogeneity or differential protein processing of FHA between SO3287-99 and KM22. There were no detectable differences in the mobility of pertactin (Fig. 3, panel A) or ACT (Fig. 3 Panel C) between KM22 and SO3287-99 under the described conditions.

When blots of whole cell extracts were incubated with a 10⁻¹ dilution of serum from the *B. bronchiseptica*-infected sea otter, numerous immunogenic proteins of up to approximately 95 kD were detected that were not apparent using the same dilution of serum obtained from a *B. bronchiseptica*-negative sea otter. A heavily stained band of roughly 40 kD present in extracts from SO3287-99 was not observed in extracts from KM22, suggesting possible antigenic heteroge-

Table 1. Hemagglutination titers.

Strain	Origin of RBCs			
	Bovine	Equine	Porcine	Ovine
SO3287-99	2	—	4	—
KM22	4	—	8	—

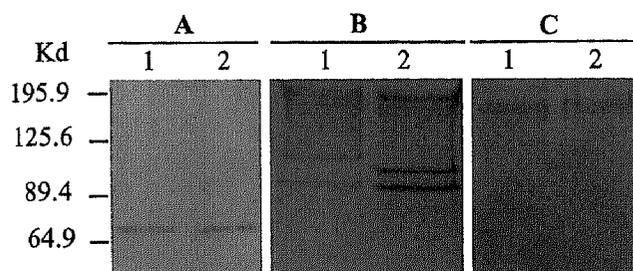


Figure 3. Western blots of extracts enriched in outer membrane proteins (panels A and B) or whole cell extracts (panel C) obtained from KM22 (lane 1) or the sea otter isolate (lane 2), incubated with monoclonal antibodies specific for pertactin (panel A), FHA (panel B), or ACT (panel C).

neity in that protein. Staining was not due to binding of Protein A because no *B. bronchiseptica* proteins were detectable when sea otter serum was omitted from the procedure (data not shown). Only a few immunogenic proteins were apparent when blots of extracts enriched in outer membrane proteins were incubated with serum from the *B. bronchiseptica*-infected sea otter. There was no difference in reactivity between KM22 and SO3287-99. No staining was observed in blots using serum from a *B. bronchiseptica*-negative sea otter.

This is the first report of *B. bronchiseptica* from a southern sea otter and the first report of ribotype-4 *B. bronchiseptica* obtained from any marine mammal. No additional cases of *B. bronchiseptica* pneumonia have been reported in captive or free-living sea otters since this isolate was obtained in November, 1999. Ribotype 4 of *B. bronchiseptica* is commonly isolated from domesticated dogs and cats.¹³ Ribotype 19 has previously been isolated exclusively from seals in Europe during the 1988 phocine morbillivirus outbreak.¹⁵

Bordetella bronchiseptica in dogs is a commonly isolated secondary opportunistic pathogen found in conjunction with canine distemper virus. No evidence of morbilliviral infection was detected based on histopathology and IHC screening in this otter. However, the effectiveness of screening for morbilliviral infections in otters is presently unknown.

Transmission of *B. bronchiseptica* typically occurs by direct animal contact and aerosols. Environmental reservoirs for *B. bronchiseptica* have been suggested to exist, including soil and freshwater habitats.^{9,11} However, the likelihood of a marine reservoir for *B. bronchiseptica* has not been reported. How this otter became infected with *Bordetella* is unknown. On the basis of the ribotype of this isolate, the possibility of transmission from a terrestrial animal (i.e., a dog) must be considered. It is possible that the severe facial trauma in this otter was not related to breeding behavior but was the result of a dog attack on an otter hauled

out near the water's edge. Although facial trauma is often associated with mating behavior in sea otters, the severity of the injury in this case is unusual. However, the wound appeared acute (approximately 24–36 hours old), whereas the pneumonia appeared acute to sub-acute (2–7 days old). Therefore, it is possible that the 2 lesions occurred independently.

In conclusion, it is unlikely that *B. bronchiseptica* is endemic in southern sea otters because routine cultures of more than 150 fresh dead otters have to date not revealed additional isolates. The use of *Pvu* II ribotyping in this case may provide clues as to the potential source of infection in this animal. Unlike the previous study in seals, where a novel clone of *B. bronchiseptica* was found to be circulating within the seal population in Europe,¹⁵ ribotyping results from the southern sea otter isolate strongly suggest the possibility of direct cross-species transmission of *B. bronchiseptica* between terrestrial and marine mammals.

Acknowledgements. The expert technical assistance of Pamala Beery, National Animal Disease Center, and Viviana Wong, University of California, Davis, is gratefully acknowledged.

Sources and manufacturers

- HistoMark Red, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD.
- Hardy Diagnostics, Santa Maria, CA.
- Microbial Identification System (MIS), Microbial ID, Inc., Newark, DE.
- Difco Laboratories, Detroit, MI.
- Corning Incorporated, Acton, MA.
- Puregene DNA Isolation Kit, Genra Systems, Minneapolis, MN.

References

- Ackermann MR, Register KB, Gentry-Weeks C, et al.: 1997, A porcine model for the evaluation of virulence of *Bordetella bronchiseptica*. *J Comp Pathol* 116:55–61.
- Baker JR, Ross HM: 1992, The role of bacteria in phocine distemper. *Sci Total Environ* 115:9–14.
- Brennan MJ, Li ZM, Cowell JL, et al.: 1988, Identification of a 69-kilodalton nonfimbrial protein as an agglutinin of *Bordetella pertussis*. 1988. *Infect Immun* 56:3189–3195.
- Doucet JP, Murphy BJ, Tuana BS: 1990, Modification of a discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide gel system for minigel electrophoresis. *Anal Biochem* 190:209–211.
- Foot JO: 1970, Nose scars in female sea otters. *J Mammal* 51: 621–622.
- Heje N-I, Henriksen P, Aalbæk B: 1991, The seal death in Danish waters 1988. *Acta Vet Scand* 32:205–210.
- Hewlett EL, Gordon VM, McCaffery JD, et al.: 1989, Adenylate cyclase toxin from *Bordetella pertussis*. Identification and purification of the holotoxin molecule. *J Biol Chem* 264:19379–19384.
- Leininger E, Probst PG, Brennan MJ, Kenimer JG: 1993, Inhibition of *Bordetella pertussis* filamentous hemagglutinin-mediated cell adherence with monoclonal antibodies. *FEMS Microbiol Lett* 106:31–38.
- Mitscherlich E, Marth EH: 1984. Bacteria and rickettsiae im-

- portant in human and animal health. *In: Microbial survival in the environment*, pp. 45–47. Springer-Verlag, Berlin, Germany.
10. Munro R, Ross H, Cornwell C, Gilmour J: 1992, Disease conditions affecting common seals (*Phoca vitulina*) around the Scottish mainland, September–November 1988. *Sci Total Environ* 115:67–82.
 11. Porter JF, Wardlaw AC: 1993, Long-term survival of *Bordetella bronchiseptica* in lakewater and in buffered saline without added nutrients. *FEMS Microbiol Lett* 110:33–36.
 12. Register KB, Ackermann MR: 1997, A highly adherent phenotype associated with virulent Bvg⁺-phase swine isolates of *Bordetella bronchiseptica* grown under modulating conditions. *Infect Immun* 65:5295–5300.
 13. Register KB, Boisvert AM, Ackermann MR: 1997, Use of ribotyping to distinguish isolates of *Bordetella bronchiseptica*. *Int J Syst Bacteriol* 47:678–683.
 14. Register KB, Magyar T: 1999, Optimized ribotyping protocol applied to Hungarian *Bordetella bronchiseptica* isolates; identification of two novel ribotypes. *Vet Microbiol* 69:277–285.
 15. Register KB, Sacco RE, Foster G: 2000, Ribotyping and restriction endonuclease analysis reveal a novel clone of *Bordetella bronchiseptica* in seals. *J Vet Diagn Invest* 12:535–540.

J Vet Diagn Invest 15:574–576 (2003)

A case of yellow fever in a brown howler (*Alouatta fusca*) in Southern Brazil

Eliza Simone Viégas Sallis, Vera Lúcia Reis Souza de Barros, Shana Letícia Garmatz, Rafael Almeida Figuera, Dominguita Lühers Graça¹

Abstract. Many brown howlers (*Alouatta fusca*) have died in a 3-month period in a subtropical forest in Southern Brazil. One was examined after a systemic illness. According to clinical signs, and necropsy and histopathology findings, yellow fever virus (YFV) infection was suspected. Tissue sections from liver, kidney, and lymphoid organs were screened by immunohistochemistry for YFV antigens. Cells within those tissues stained positively with a polyclonal antibody against YFV antigens (1:1,600 dilution), and yellow fever was diagnosed for the first time in the brown howler in the area.

Yellow fever virus (YFV) infection is an acute arthropod-borne Flavivirus (family *Togaviridae*) infection that causes fever, jaundice, albuminuria, and hemorrhage. It occurs in 2 forms: urban and sylvan. There are 2 types of endemic areas: humid forests and emerging zones where urban and sylvan forms intermingle.³ The vectors are *Aedes aegypti* in urban areas,⁹ *A. albopictus* in suburban areas,¹⁰ and tree-hole-breeding mosquitoes (*Haemagogus* spp.) in the forests.⁹ The virus circulates in the forests in mosquito vectors causing scattered epizootics in nonimmune monkeys. Alternatively, transmission may be vertical from the female mosquito to her offspring, allowing virus survival from one rainy season to the next in *A. aegypti* eggs.³ Yellow fever remains endemic in many regions of Africa and South America, despite the existence of an effective vaccine.^{2,9} Currently, YFV infection may be confused with other similar hemorrhagic conditions.^{2,4,9}

In humans, severe necrotic lesions are seen at autopsy² in many organs, particularly the liver. Microscopically, YFV infection is characterized by midzonal necrosis of the liver with microvesicular fatty change

of hepatocytes, the presence of apoptotic bodies (Councilman bodies), renal tubular degeneration, and splenic lymphoid necrosis.^{5,6,8}

Up to 80 brown howlers (*Alouatta fusca*) are suspected to have died from March 2001 to May 2001 at the border between Brazil and Argentina. People of the subtropical forest of the area reported that many dead monkeys had fallen from the trees. They observed that the monkeys had yellow mucosae and skin. A yellow fever outbreak was suspected. One of the dead monkeys was necropsied in May 2001 at the Department of Pathology, Universidade Federal de Santa Maria, Southern Brazil.

The necropsied brown howler was a thin, young adult female monkey in poor body condition. All mucosae and large vessel intimae and the liver were jaundiced. The right kidney had an irregular area at the surface, and the spleen had uneven borders. The stomach was full of nondigested green leaves, fibers, and seeds. The intestines contained feces coated in dry, yellow mucus. The urinary bladder contained yellow urine with white floccular strands. Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μ m and stained with hematoxylin and eosin (HE). By light microscopy, massive coagulation necrosis of most hepatocytes and fatty degeneration of the remaining cells was observed in the liver (Fig. 1). Scattered apoptotic hepatocytes were present. Renal tubular cells had de-

From the Departamento de Patologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, Brazil (Sallis, Garmatz, Figuera, Graça), and the Instituto Evandro Chagas, Rod. BR-316, Km 7, 67030-000 Ananindeua, PA, Brazil (de Barros). ¹ Corresponding author.