Case Report
J Vet Intern Med 2010;24:633–638

Severe Hepatitis Associated with Acute *Ehrlichia canis* Infection in a Dog

M.E. Mylonakis, M. Kritsepi-Konstantinou, J.S. Dumler, P.P.V. P. Diniz, M.J. Day, V.I. Siarkou, E.B. Breitschwerdt, V. Psychas, T. Petanides, and A.F. Koutinas

An 8-year-old, intact male German Shepherd dog was referred because of anorexia, intermittent vomiting, and diarrhea of 5 days' duration. The dog was housed outdoors and was incompletely vaccinated (booster interval in excess of 5 years for parvovirus, distemper, adenovirus, and *Leptospira* spp.). Physical examination (including monocular indirect ophthalmoscopy) identified lethargy, weakness, moderate pain on anterior abdominal palpation, dehydration, halitosis, and fever (40.4°C).

CBC[a] was unremarkable, whereas the important serum biochemical findings included hypoalbuminemia (2.1 g/dL; reference range, 2.5–3.5 g/dL), normoglobulinemia (3.9 g/dL; reference range, 3–4.5 g/dL), hyperbilirubinemia (2.1 mg/dL; reference range, 0.3–0.8 mg/dL), and increased alkaline phosphatase (ALP) (1,300 U/L; reference range, 50–210 U/L) and alanine aminotransferase (ALT) (160 U/L; reference range, 10–34 U/L) activities. Analysis of an orange-colored urine sample obtained by cystocentesis disclosed an average of 2 granular casts/low power field and a urine protein-to-creatinine ratio of 2.9 (reference interval, <0.5); urine culture failed to grow bacteria. On admission, a Giemsa-stained buffy coat smear was negative for *Ehrlichia* sp., *Babesia* sp., *Hepatozoon canis*, *and Mycoplasma* sp. organisms (1,000 microscopy fields). Serum was tested for *Leishmania infantum* and *Babesia canis* antibodies by indirect immunofluorescence assays (IFA), whereas *Ehrlichia canis* antibodies were tested by an in-office ELISA[b] test. All 3 tests were negative. The dog was positive for *Dirofilaria immitis* antigens[c] but negative on a modified Knott's test for microfilaria. Thoracic radiography was unremarkable, but moderate hepatomegaly and splenomegaly were evident on abdominal radiographs and confirmed by abdominal ultrasonography (enlarged and diffusely hypoechoic liver). Leptospirosis was suspected, based on epidemiologic (endemic disease in Greece), historical, and clinical data and the fact that all the infectious disease tests described above were negative. Dirofilariasis (stage 1) was not considered a contributory factor for the clinical presentation of this dog. Pending microscopic agglutination test (MAT) serology results for *Leptospira* spp., the dog was hospitalized and treated with crystalloids[d] (60 mL/kg IV daily), ampicillin[e] (20 mg/kg IV q8h), enrofloxacin[f] (10 mg/kg SC q24h), ursodeoxycholic acid[g] (15 mg/kg PO q24h), ranitidine[h] (2 mg/kg IV q12h), and sucralfate[i] (1 g/30 kg PO q12h). Because no clinical or biochemical improvement was noticed after 5 days of hospitalization, a diagnostic laparotomy was performed to obtain wedge liver biopsies. Apart from mild hepatomegaly and firm consistency of hepatic parenchyma, no other abnormalities were noticed. Imprint smears were made of hepatic tissue and Giemsa-stained for cytological examination. Formalin-fixed and frozen liver tissue was retained for future polymerase chain reaction (PCR) and histopathology.

Impression cytology of the liver identified several round-to-oval and occasionally polyhedral hepatocytes with an eccentrically placed round nucleus (occasional cells were binucleated) and slightly basophilic cytoplasm that were distributed individually or in clusters among a slightly hemorrhagic background. Dark green intracytoplasmic granules consistent with bile and solid green to black bile casts were observed between contiguous hepatocytes. There were numerous mature lymphocytes, with fewer plasma cells, macrophages, and nondegenerate neutrophils dispersed among the hepatocytes (Fig 1). Several dark-blue cytoplasmic inclusions consistent with *Ehrlichia* sp. morulae were observed in lymphocytes and macrophages (Fig 1). There was also mild extramedullary

Abbreviations:

ALP	alkaline phosphatase
ALT	alanine aminotransferase
BM	bone marrow
CME	canine monocytic ehrlichiosis
IFA	indirect immunofluorescence assay
MAT	microscopic agglutination test
PA	postadmission
PCR	polymerase chain reaction

From the Companion Animal Clinic (Mylonakis, Petanides, Koutinas), the Diagnostic Laboratory (Kritsepi-Konstantinou), the Laboratory of Microbiology and Infectious Diseases (Siarkou), the Laboratory of Pathology (Psychas), Faculty of Veterinary Medicine, Aristotle University of Thessaloniki (AUTh), Greece; the Department of Pathology, Division of Medical Microbiology, The Johns Hopkins University School of Medicine, Baltimore, MD (Dumler); the College of Veterinary Medicine, Western University of Health Sciences, Pomona, CA (Diniz); the Intracellular Pathogens Research Laboratory, College of Veterinary Medicine, North Carolina State University, Raleigh, NC (Breitschwerdt); and the Division of Veterinary Pathology, Infection and Immunity, School of Clinical Veterinary Science, University of Bristol, UK (Day). *This work was undertaken in the Companion Animal Clinic, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. An oral presentation of this case was presented by the first author at the 11th ECVCP Congress, October 7–9, 2009, Thessaloniki, Greece (proceedings p. 166).*

Corresponding author: Mathios E. Mylonakis, DVM, 18 Kyprou Str., 55133, Thessaloniki, Greece; e-mail: mmylonak@vet.auth.gr.

Submitted September 9, 2009; Revised February 2, 2010; Accepted February 18, 2010.

10.1111/j.1939-1676.2010.0501.x

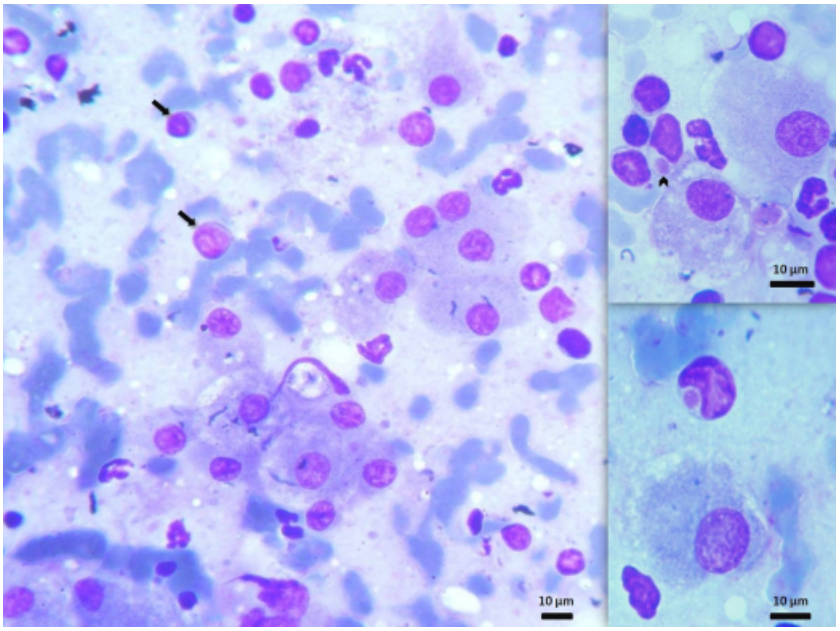

Fig 1. Photomicrograph of liver impression cytology. Numerous small lymphocytes (arrows) are seen adjacent to single or clustered hepatocytes (Giemsa stain). The inset in the upper right corner contains lymphocytes and neutrophils adjacent to 2 hepatocytes. A cytoplasmic *Ehrlichia* sp. morula is seen in a lymphocyte (arrowhead). In the lower right corner inset, an *Ehrlichia* sp. morula is seen in a lymphocyte adjacent to a hepatocyte. Bar = 10 µm.

hematopoiesis, identified by occasional metarubricytes, metamyelocytes, and megakaryocytes. Bone marrow (BM) cytology performed the day after liver cytology was normocellular, with normal appearing hemopoietic lineages, mild plasmacytosis, and no evidence of *Ehrlichia* inclusions or other infectious agents.

Histopathology of liver tissue stained with hematoxylin and eosin disclosed moderate portal fibrosis with bile duct proliferation, dilatation of thin-walled vessels and subtle portal to portal bridging fibrosis. There also was a mild mixed inflammatory infiltrate associated with the portal areas that included lymphocytes, plasma cells, macrophages, neutrophils, and eosinophils (Fig 2). Mild portal hemorrhage accompanied by occasional hemosiderophages was present. There was mild diffuse microvesicular vacuolation of the hepatocyte cytoplasm and generalized vascular congestion. Overall, the histopathological findings were indicative of portal hepatitis. Immunohistochemistry was performed on the formalin-fixed liver tissue as previously described[1] except that *E. canis* monospecific polyclonal antibody to *E. canis* gp36 (36 kDa glycoprotein) antigen was used (The Johns Hopkins University School of Medicine, Baltimore, MD). As positive controls, lung tissue from a known *E. canis*-infected dog and splenic tissue from a human patient infected with *Ehrlichia chaffeensis* were used (Fig 3). Several cells in the hepatic tissue, presumably monocytes and lymphocytes, were infected with *Ehrlichia* spp. (Fig 3).

At day 7 postadmission (PA), a CBC obtained before the institution of doxycycline treatment indicated moderate thrombocytopenia (130,000/µL; reference range 200,000–500,000/µL) and buffy coat smears were negative for *Ehrlichia* morulae (2,000 microscopy fields). However, *E. canis* 16S rDNA was amplified by PCR assays from a whole blood sample (Laboratory of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, AUTh) obtained on day 7 PA. In addition, *E. canis* DNA subsequently was amplified and sequenced from BM aspirate material and frozen-preserved liver tissue (obtained on days 7 and 6 PA, respectively) (Intracellular Pathogens Research Laboratory, College of Veterinary Medicine, NCSU).[2,3] The sequences determined by direct sequencing of the approximately 1.4 kb PCR amplicons (nearly entire length of ehrlichial 16S rDNA)[3] or those obtained from clones of the 431 bp PCR amplicons from BM (*E. canis*-specific 16S rDNA segment) into pGem-T Easy vector[j] were 99.7% identical with each other (1 base pair different in 364 bp, with primers excluded from analysis), as well as to *E. canis* Greek strains previously characterized (GenBank accession numbers EF011110 and EF011111).[3] DNA sequence of *E. canis* from the liver was 100% similar to the Greek strains mentioned before. DNA from *Bartonella* sp. was not amplified from the liver and BM after applying a 1-step conventional PCR.[4] Also, MAT for *Leptospira* spp. was negative.

Treatment with doxycycline[k] (5 mg/kg PO q12h for 4 weeks) was instituted 24 hours after visualization of morulae in impression smears (day 7 PA). Fever abated within 12 hours and clinical resolution of all clinical signs occurred within 48 hours after initiating doxycycline therapy. Serial serum biochemical analyses showed hypoalbuminemia (2.0 g/dL; reference range 2.5–3.5 g/dL), hyperbilirubinemia (1.1 mg/dL; reference range 0.3–0.8 mg/dL), increased ALP (761 U/L; reference range

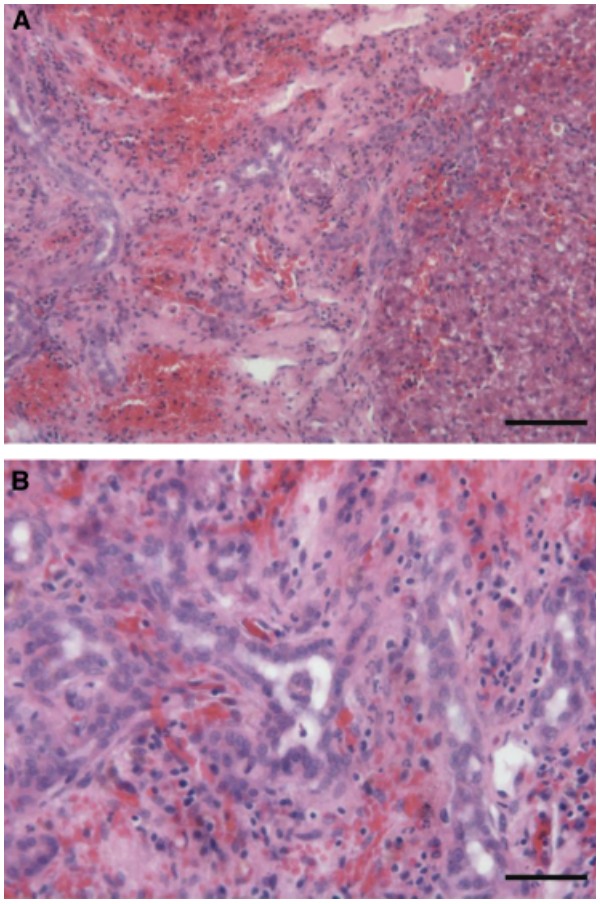

Fig 2. (A) Photomicrograph of liver histopathology showing portal fibrosis, bile duct proliferation, hemorrhage, and inflammation. There is mild microvesicular vacuolation of the cytoplasm of adjacent hepatocytes (hematoxylin and eosin stain). Bar = 200 μm. (B) Higher magnification of affected portal area showing mixed neutrophilic and mononuclear inflammation. Bar = 100 μm.

50–210 U/L) and ALT (238 U/L; reference range 10–34 U/L) activities at days 8 and 20 (ALP, 322 U/L; ALT, 83 U/L) PA; all biochemical abnormalities had resolved by day 40 PA. CBC results at days 20 and 40 PA were within reference ranges. Serological results with in-office ELISA and IFA tests were negative for *E. canis* antibodies at 2 and 4 weeks PA, respectively. The dog was seroreactive for *E. canis*, however, at 6 weeks PA (IFA titer, 1 : 800; laboratory cut-off value, 1 : 100). Retrospectively, IFA for *Rickettsia rickettsii*, performed in paired serum samples from days 7 and 40 PA, was positive (titers 1 : 256 and 1 : 512, respectively; laboratory cut-off value, $\geq$ 1 : 64), but DNA from spotted fever group rickettsiae was not amplified from the liver and BM after applying a PCR assay as described previously.[5] The split-dose (a single injection of 2.5 mg/kg IM, followed 1 month later, by 2 injections of the same dose, 24 hours apart) melarsomine[1] protocol for *D. immitis* also was instituted after completion of doxycycline therapy. After the last clinicopathological evaluation (day 40 PA), regular phone communications indicated that the animal has remained clinically healthy during the 4-year follow-up period.

An *E. canis*-associated severe hepatitis was suspected in this dog, based on clinical findings, liver cytology, histopathology, immunohistochemistry, PCR amplification of *E. canis* DNA from blood, BM, and liver by 2 different laboratories, and the temporal relationship of the clinical response to initiation of doxycycline treatment. Historically, mild-to-moderate increases in liver enzyme activities and liver histopathology frequently have been recognized in canine monocytic ehrlichiosis (CME),[6–8] but *E. canis*-associated hepatopathy is extremely rare as a predominant clinical manifestation, especially in the acute phase of the disease. Although clinical staging in natural CME is not straightforward, the acute onset of illness in this dog in association with the seroconversion (6 weeks PA) and a normocellular BM seem to support the acute nature of *E. canis* infection.[9] Severe hepatic disease has been reported uncommonly in pancytopenic dogs with advanced *E. canis*-induced BM aplasia, which could be attributed to anemic hypoxia, intrahepatic hemorrhage and occasionally, to secondary bacterial sepsis. Nevertheless, even in these chronic cases, liver is not the sole or target organ.[10,11] In this dog, the histological diagnosis was portal hepatitis, based on a mixed inflammatory infiltration, portal fibrosis and cholestasis; the latter was documented by hepatic cytology (visualization of bile casts in canaliculi) along with hyperbilirubinemia and increased ALP activity.[12] However, no histopathologic evidence of cholestasis was noticed, probably because of the embedding procedure.[13] The moderate fibrosis seen in this dog has not been previously reported in experimentally or naturally infected dogs[6] and therefore is difficult to explain in the context of the acute CME; therefore, the possibility that the *E. canis*-associated hepatopathy in this dog occurred in addition to a preexisting liver insult cannot be ruled out. In 2 previous experimental studies of CME, portal infiltration with lymphocytes, plasma cells, macrophages, and focal accumulations of reticuloendothelial cells compressing adjacent hepatocytes,[14] or centrilobular fatty degeneration and mild-to-moderate perivascular with periportal mononuclear cell infiltration have been described.[15] Also, in a retrospective pathological study of naturally infected dogs, many of which were assumed to be chronically infected, centrilobular degeneration or necrosis and portal plasmacytosis were found.[6] Despite the fact that none of these dogs had overt liver disease, mixed cell or lymphoplasmacytic portal inflammation was a common histopathological finding. Interestingly, severe *E. chaffeensis*-associated hepatitis in acutely infected people has been associated with similar hepatic pathology (ie, mixed cell portal infiltration, cholestasis, and focal hepatic necrosis), which, similarly to this dog, may occur before seroconversion.[1,16,17] The fact that hepatitis was disproportionally severe in human patients with few or no *E. chaffeensis* morulae in leukocytes, hepatocytes, or biliary structures has raised suspicions that pathogenesis of *E. chaffeensis* hepatitis is related to an overzealous host immune response to the organism, rather than the direct cytopathic

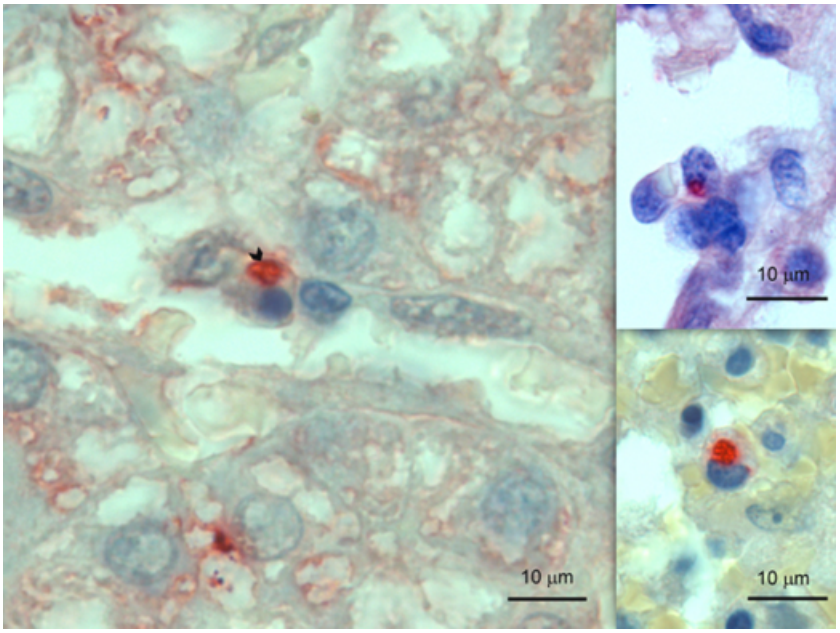

Fig 3. Immunohistological demonstration of *Ehrlichia canis* from the liver of a dog with acute portal hepatitis and monocytic ehrlichiosis. *E. canis* morula (arrowhead) within the cytoplasm of a mononuclear cell. Upper right inset: *E. canis* morula within the cytoplasm of a mononuclear cell from lung tissue of a known *E. canis*-infected dog (*E. canis* positive control). Lower right inset: *E. chaffeensis* in the cytoplasm of a mononuclear cell from the spleen of a human patient (*E. chaffeensis* positive control). *E. canis* immunohistochemistry with hematoxylin counterstain. Bar = 10 μm.

effect of the organism itself. A similar pathogenesis also could apply to CME.[1]

Upon admission, the dog did not show many of the typical clinical and clinicopathological features of CME, such as thrombocytopenic bleeding tendency, peripheral lymphadenomegaly, and positive antibody titer to *E. canis*. On the other hand, documentation of an acute febrile illness accompanied by hepatic and renal clinicopathologic abnormalities raised the suspicion of leptospirosis.[18] However, the ensuing treatment with ampicillin and enrofloxacin failed to improve the clinical condition of the dog. Notably, in a previous study, the lack of efficacy of enrofloxacin in dogs experimentally infected with *E. canis* also was documented.[19]

Thrombocytopenia, which is common in CME, appeared only transiently in this dog (at day 7 PA), emphasizing the fact that ehrlichiosis cannot be excluded because of a normal platelet count.[6] Hypoalbuminemia, a consistent finding in CME,[7,11] was attributed to protein-losing nephropathy, liver disease, vascular injury, or some combination of these. Serum albumin concentrations were normalized by day 20 PA, along with the improvement of liver disease but the proteinuria was not further evaluated. Although seroconversion is used to document acute ehrlichiosis in dogs and humans, it may lag behind clinical expression of the disease[9]; the same could have applied in this dog because seroconversion did not occur until 6 weeks PA.

The first evidence supporting *E. canis* infection in this dog was obtained after a careful review of liver cytology, in which several morulae were found. In contrast, buffy coat smears (which were shown in 1 study[20] to be of high diagnostic sensitivity in acute CME) were repeatedly negative in this dog between days 1 and 7 PA. It is hypothesized that this dog may have been infected with a hepatotropic strain of *E. canis*. In this respect, use of a quantitative real-time PCR assay could provide a more efficient demonstration of ehrlichial density in the liver, as compared with blood, BM, or other tissues, because the conventional PCR assays used in both laboratories provide only qualitative evidence (DNA detected or not detected) of the presence of *E. canis* in the various tissues.[21] To our knowledge, this is the first case report describing immunohistological demonstration of *E. canis* morulae similar to what has been described in human monocytic ehrlichiosis and may be useful in establishing a diagnosis of CME.[1,22] The numerous morulae found on hepatic cytology and immunohistochemistry of this dog, together with the detection of *E. canis* DNA in the liver and the observation of morulae in hepatic imprints of acutely infected dogs,[14] suggest that the liver should be considered as a potential target organ for application of PCR testing for epidemiological studies and posttreatment monitoring.[23,24]

The possibility of coinfection with canine leishmaniosis, bartonellosis, and spotted fever group rickettsiosis was ruled out with the aid of serology and BM cytology (leishmaniosis), BM and liver PCR (*Bartonella* spp.), and BM and liver PCR (spotted fever group rickettsiosis), because these diseases have been associated with liver disease either symptomatic or asymptomatic.[25–27] Based on our experience, in canine leishmaniosis the associated liver disease is asymptomatic in most cases,[26] with only a few dogs showing chronic hepatitis, liver failure or both

which usually responds to antileishmanial and supportive treatment. The different sequential IFA titers for *R. rickettsii* were interpreted as being identical, as the criterion for seroconversion (ie, a 4-fold rise in antibody titer) was not achieved.[27] Interestingly, in a previous study in Greece, 10/19 dogs with CME had serological evidence of exposure to *R. conorii* (the agent of Mediterranean spotted fever), but PCR failed to amplify the rickettsial DNA from any of the dogs,[11] suggesting that Greek dogs with CME frequently are exposed to, but rarely persistently infected by, a rickettsial agent. The authors also cannot rule out that this dog might have been chronically infected by *E. canis*,[28] and that the dramatic response to treatment was the result of eradication of another doxycycline-responsive agent. However, the seroconversion documented in this dog strongly suggests recent infection. Dirofilariosis was considered an incidental finding in this dog, because there was no historical, clinical, or radiographic evidence of clinical disease and most importantly, the marked clinical and biochemical improvement preceded the institution of adulticide treatment with melarsomine.

In conclusion, although a cause-and-effect relationship cannot be definitely established, this report provides strong evidence that acute CME may induce symptomatic hepatitis as the predominant clinical manifestation. It is therefore suggested that CME should be included in the differential diagnosis when dogs are admitted with fever and laboratory evidence of hepatic disease, especially in the endemic areas for the disease.

Footnotes

[a] VetABC, Scil Animal Care Company, Viernheim, Germany
[b] ImmunoComb, Biogal-Galed, Kibbutz Galed, Israel
[c] Snap Canine Heartworm PF; IDEXX Laboratories Inc, Westbrook, ME
[d] Lactated Ringers, Vioser, Trikala, Greece
[e] Ampicillin, Pentrexyl, Bristol-Myers-Squibb, New York, NY
[f] Baytril, Bayer, Leverkusen, Germany
[g] Ursofalk, Galenica, Athens, Greece
[h] Zantac, Glaxo-S.K., Athens, Greece
[i] Peptonorm, Uni-Pharma, Athens, Greece
[j] Promega, Madison, WI
[k] Ronaxan, Merial, Lyon, France
[l] Immiticide, Merial

Acknowledgments

The authors thank Dr Jere McBride, Department of Pathology, University of Texas Medical Branch, Galveston, for generously providing the polyclonal antibodies to *E. canis* glycoproteins.

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