Detection of Bartonella henselae and Bartonella clarridgeiae DNA in hepatic specimens from two dogs with hepatic disease

Tracey N. Gillespie, DVM; Robert J. Washabau, VMD, PhD, DACVIM; Michael H. Goldschmidt, BVMS, DACVP; John M. Cullen, VMD, PhD, DACVP; Allison R. Rogala, BS; Edward B. Breitschwerdt, DVM, DACVIM

Peliosis hepatis has been associated with Bartonella henselae infection in dogs and people, and B henselae has increasingly been recognized as an important cause of granulomatous hepatitis in people.

Bartonella organisms may represent a primary cause of hepatic disease in dogs or may be opportunistic invaders in dogs with preexisting hepatic disease.

A 4-year-old spayed female Basset Hound (case 1) was referred to the Veterinary Hospital of the University of Pennsylvania with a 6-month history of recurrent fever, anorexia, and weight loss. Previous serum biochemical testing had identified high hepatic enzyme activities. Three months prior to referral, histologic examination of a hepatic biopsy specimen revealed severe pyogranulomatous inflammation with moderate telangiectasia but without evidence of a causative agent. Despite treatment with various antimicrobials (amoxicillin, enrofloxacin, and metronidazole), vitamins E and C, ursodeoxycholate, and milk thistle, clinical signs persisted and hepatic enzyme activities continued to increase.

On physical examination at the veterinary hospital, the dog appeared mildly icteric; results of the remainder of the examination were unremarkable. The margins of the liver were not palpable. Complete blood count, serum biochemical analyses, coagulation profile, baseline plasma ammonia determination, thoracic and abdominal radiography, and abdominal ultrasonography were performed. Hematologic abnormalities included leukocytosis (24.9 \times 10^3 \text{ cells/µL}; reference range, 6.7 to 18.3 \times 10^3 \text{ cells/µL}) and mature neutrophilia (22 \times 10^3 \text{ cells/µL}; reference range, 3.6 to 12.5 \times 10^3 \text{ cells/µL}). Biochemical abnormalities included high serum alkaline phosphatase (ALP) activity (598 U/L; reference range, 20 to 150 U/L), high alanine aminotransferase (ALT) activity (332 U/L; reference range, 10 to 118 U/L), hyperbilirubinemia (1.5 mg/dL; reference range, 0.1 to 0.6 mg/dL), hypocholesterolemia (117 mg/dL; reference range, 125 to 270 mg/dL), and low blood urea nitrogen concentration (6 mg/dL; reference range, 7 to 25 mg/dL). Prothrombin time (11.6 seconds; 11% of control patient value), activated partial thromboplastin time (12.8 seconds; 6% of control patient value), and serum albumin concentration (2.6 g/dL; reference range, 2.3 to 3.9 g/dL) were within reference limits. The baseline plasma ammonia concentration (10 µmol/L; reference range, 10 to 35 µmol/L) was normal. On abdominal radiographs, cranial displacement of the gastric axis and a small hepatic silhouette were seen; these signs were considered suggestive of a generalized decrease in liver volume.

During abdominal ultrasonography, the liver appeared to be of mixed echogenicity with ill-defined hypoechogenic regions throughout the hepatic parenchyma. Extrahepatic biliary structures (eg, hepatic ducts, gallbladder, cystic duct, and common bile duct), pancreas, and intestine were ultrasonographically normal. Thoracic radiographs were unremarkable.

The dog was sedated with propofol and diazepam IV in preparation for percutaneous ultrasound-guided liver biopsy. Tissue specimens were obtained from the right lateral liver lobe with a 14-gauge biopsy needle and submitted for cytologic evaluation, routine histologic evaluation (H&E stain), acid-fast staining, and bacterial and fungal culture. Cytologically, aspirates of the liver contained many healthy mature neutrophils, moderate numbers of small lymphocytes and macrophages, and occasional plasma cells, consistent with a mixed inflammatory cell infiltrate. Hepatocyte morphology appeared normal, and there were occasional canalicular bile plugs. No etiologic agents were seen. Histologic examination of liver biopsy specimens revealed granulomas consisting of epithelioid cells admixed with a few neutrophils distributed diffusely throughout the specimen (Fig 1). Fibrosis and bile duct proliferation were evident in some portal areas, with bridging between portal areas. In addition, single cell necrosis of hepatocytes was seen in areas unrelated to sites of granulomatous inflammation. No etiologic agents were observed in the stained sections. Results of examination of sections stained with Warthin-Starry stain or with acid-fast stain for Mycobacterium species were negative. Aerobic, anaerobic, mycobacterial, and fungal cultures failed to yield any growth. Serologic testing did not reveal antibodies against any Leptospira serovars, except for L icterohemorrhagiae (1:100; reference range, < 1:400). The dog was seronegative for infection with Toxoplasma spp, Neospora spp, and fungal organisms, and antib-
ies could not be demonstrated for *Ehrlichia canis*, *E risticii*, *Rickettsia rickettsia*, or *Borreia burgdorferi*.

The dog recovered from sedation without complications, and treatment with clavulanic acid-amoxicillin (12 mg/kg [5.5 mg/lb], PO, q 12 h), metronidazole (12 mg/kg, PO, q 12 h), and enrofloxacin (10 mg/kg [4.5 mg/lb], PO, q 24 h) was instituted, pending histology and culture results. During the next 10 days, the dog continued to deteriorate clinically. Vomiting developed but resolved when administration of clavulanic acid-amoxicillin and metronidazole was discontinued. Treatment with prednisone (1 mg/kg [0.43 mg/lb], PO, q 12 h) was instituted, and there was transient resolution of the dog's anorexia. However, after 10 days, the dog developed severe ascites, a tense abdomen, and vomiting. Furosemide (1 mg/kg, PO, q 12 h) and spironolactone (1 mg/kg, PO, q 12 h) were prescribed, and treatment with prednisone was gradually discontinued over 2 weeks. Within 2 weeks, the ascites resolved, and diuretic treatment was discontinued.

Because infection with *Bartonella* spp has been associated with granulomatous disease (including hepatitis) in people, serum was tested with an indirect immunofluorescent antibody (IFA) test incorporating *Bartonella vinsonii* (berkhoffii) antigens and with a western immunoblot analysis incorporating *Bartonella henselae* antigens. Antibodies were not detected with the IFA test at a screening dilution of 1:16, indicating that the dog had probably not been exposed to *B vinsonii* (berkhoffii). However, the western immunoblot pattern was indicative of prior exposure to *Bartonella* organisms, potentially *B henselae*. A polymerase chain reaction (PCR) assay, performed as described, was used on 2 occasions to amplify DNA extracted from formalin-fixed, paraffin-embedded hepatic tissue. In both instances, PCR amplification yielded a 172-base pair band, indicating that hepatic tissue contained *B henselae* DNA. To confirm results of the initial PCR assays, DNA was again extracted, approximately 8 months later, from the same hepatic biopsy specimen and amplified with the PCR assay. Amplicons (160 base pairs) were sequenced, and the sequence was found to be identical to a portion of the published DNA sequence for *B henselae* (GenBank accession No. L35101).

After results of serologic testing for *Bartonella* infection and the initial PCR assays became available, treatment with azithromycin (5 mg/kg [2.3 mg/lb], PO, q 48 h) was instituted for 10 weeks for treatment of presumptive *B henselae*-induced granulomatous hepatitis. Within 4 weeks, the dog's clinical signs improved dramatically. Serum ALP activity (339 U/L) decreased, although it did not return to reference limits, and serum ALT activity (100 U/L) returned to reference limits. The hypocholesterolemia (147 mg/dL) and hyperbilirubinemia (0.7 mg/dL) also resolved. Because the dog's clinical signs appeared to recur with cessation of the medication, the owner was reluctant to discontinue administration of the azithromycin. Twelve weeks after treatment with azithromycin was begun, the owner empirically elected to continue administration of azithromycin at the previous dosage and also administered metoclopramide (0.2 mg/kg [0.1 mg/lb], PO, q 12 h), ursodeoxycholate (6 mg/kg [2.7 mg/lb], PO, q 12 h), vitamin E (18 U/kg [8.2 U/lb], PO, q 24 h), and milk thistle extract (8 mg/kg [3.6 mg/lb], PO, q 24 h). Six months following hepatic biopsy, follow-up blood work was performed and revealed high serum ALP (426 U/L) and ALT (359 U/L) activities. The serum albumin (2.6 g/dL), bilirubin (0.6 mg/dL), and cholesterol (138 mg/dL) concentrations remained within reference limits, although values were decreased, compared with previous values. Following initial examination at our hospital, the dog remained substantially improved for 13 months, at which time it developed fever, anorexia, vomiting, polyuria, and polydipsia and was euthanatized at the owner's request. Although a complete necropsy was not performed, liver tissue was obtained post mortem for histologic examination and a *Bartonella* PCR assay. Histologic findings included severe bile duct proliferation, fibrosis, loss of hepatocytes, and loci of regenerative nodular hyperplasia (Fig 2). Five attempts to extract DNA for PCR amplification were not successful, presumably because of extended fixation in formalin.

A 6-year-old spayed female Doberman Pinscher...
(case 2) was found to have high hepatic enzyme activities on routine preanesthetic blood tests obtained prior to dental prophylaxis. Pertinent medical history included treatment for heartworm disease 2 years previously and a long-standing history of urinary incontinence that was well controlled with phenylpropanolamine (0.5 mg/kg [0.23 mg/lb], PO, q 12 h). The dog had received numerous homeopathic substances and vitamin supplements, including vitamin C, cod liver oil, flax oil, and a multivitamin preparation, over an extended period. Other than mild weight loss, the dog had no other clinical signs associated with the high hepatic enzyme values.

On physical examination, the dog was in good body condition and had no evidence of hepatomegaly or other hepatic abnormalities. Complete blood count, serum biochemical profile, urinalysis, determination of serum bile acids concentration, abdominal radiography, abdominal ultrasonography, and coagulation profile were performed as part of the initial medical investigation. Results of the CBC, coagulation profile, and abdominal radiography were all within reference limits or unremarkable. Results of the urinalysis were normal except for trace bilirubinuria, rare triple phosphate crystals, a few amorphous phosphate crystals, and a few RBCs. Biochemical abnormalities included high serum ALP (1,101 U/L; reference range, 5 to 131 U/L), ALT (954 U/L; reference range, 12 to 118 U/L), aspartate aminotransferase (AST; 288 U/L; reference range, 15 to 66 U/L), and γ-glutamyltransferase (GGT; 15 U/L; reference range, 1 to 12 U/L) activities and hyperbilirubinemia (0.8 mg/dL; reference range, 0.1 to 0.3 mg/dL). Preprandial serum bile acids concentration was slightly abnormal (28.1 µmol/L; reference range, 0.0 to 5.0 µmol/L), but the postprandial serum bile acids concentration was normal (8.3 µmol/L; reference range, 5.0 to 25.0 µmol/L). A diffuse, mixed echogenic pattern with hypoechoic nodules and hyperechoic strands was observed in the hepatic parenchyma during abdominal ultrasonography.

Anesthesia was induced with propofol and maintained with isoflurane, and hepatic biopsy specimens were submitted for routine histologic examination and aerobic bacterial culture. Multiple pieces of liver were submitted for routine histologic examination and aerobic bacterial culture. Histologically, hepatic specimens were characterized by moderate to severe lymphocytic hepatitis with moderate to marked bridging fibrosis, nodular regeneration, and pigmented hepatopathy. No etiologic agents were observed, and bacterial culture did not yield any growth. Hepatic tissue copper content, determined by means of dry weight analysis, was subsequently determined to be in the toxic range (3,170 ppm; reference range, 120 to 400 ppm). The histologic findings and high hepatic copper content were consistent with a diagnosis of Doberman hepatopathy.

As a recent pathologic accession, this dog's hepatic biopsy specimen had been randomly selected for PCR analysis, in conjunction with hepatic specimens from 4 other dogs with nonspecific inflammatory hepatic disease, as a presumed negative tissue control sample for the PCR assay for case 1 described in the present report. Positive control samples were derived from cultures of B henselae and B vinsonii (berkhoffii). In addition, the standard PCR mixture without DNA was assayed as a negative control sample. In an attempt to amplify a longer strand of DNA for sequence determination, a modified PCR assay developed in our laboratory that incorporated 20 pmol of primer Bart 16-23S.F (5′-TTTTTGATCTSATGAGCA-3′) and 20 pmol of primer Bart 16-23S.R (5′-CAAGCGCGCGCTCTTAAC-3′) was used. Amplification conditions included 2 minutes of denaturation followed by 60 cycles at 94°C for 1 minute, 54°C for 30 seconds, and 72°C for 1 minute. This was followed by an extension time of 10 minutes at 72°C. Bartonella DNA was not detected in the control sample without DNA or in control samples from 4 of the 5 dogs with hepatic disease when the assay was performed with published or modified primers. However, a band of approximately 400 base pairs was amplified from the hepatic biopsy specimen from the Doberman Pinscher. The DNA sequence of this band was 100% identical to the published sequence for B clarridgeiae (GenBank accession No. AF167989).

The dog was treated with prednisone at an antiinflammatory dosage (0.25 mg/kg [0.11 mg/lb], PO, q 12 h) and with azathioprine at an immunosuppressive dosage (1.8 mg/kg [0.82 mg/lb], PO, q 24 h for 3 weeks, then q 48 h). The dog also was given S-adenosyl-methionine (7.5 mg/kg [3.4 mg/lb], PO, q 24 h), vitamin E (7.5 mg/kg [3.4 mg/lb], PO, q 12 h), ursodeoxycholate (11 mg/kg [5 mg/lb], PO, q 24 h), tiotidine hydrochloride (9.25 mg/kg [4.2 mg/lb], PO, q 12 h) for copper chelation, and numerous other holistic supplements given at the owner's discretion. Blood test results obtained 6 weeks later revealed marked increases in ALP (7,880 U/L) and GGT (414 U/L) activities that were attributed to prednisone treatment. The ALT activity (577 U/L), AST activity (83 U/L), and hyperbilirubinemia (0.7 mg/dL) had improved dramatically. Treatment with phenylpropanolamine was continued as previously prescribed, but the pet owner discontinued administration of prednisone because of worsening incontinence. Following PCR detection of B clarridgeiae, the dog was treated with azithromycin (9.25 mg/kg [4.2 mg/lb], PO, q 12 h for 10 days, then q 24 h for 10 days, then q 48 h for 6 weeks). Following treatment with azithromycin for 2 weeks, laboratory testing revealed substantial decreases in ALP activity (1,482 U/L), ALT activity (221 U/L), GGT activity (16 U/L), and hyperbilirubinemia (0.4 mg/dL). The AST activity (47 U/L) had returned to reference limits. The dog did well clinically for 12 months, but then developed signs consistent with hepatic failure, including ascites, anorexia, and profound muscle wasting. Laboratory testing at this time revealed increased ALP activity (4,160 U/L), ALT activity (221 U/L), GGT activity (16 U/L), and hyperbilirubinemia (0.4 mg/dL). The AST activity (47 U/L) had returned to reference limits. The dog did well clinically for 12 months, but then developed signs consistent with hepatic failure, including ascites, anorexia, and profound muscle wasting. Laboratory testing at this time revealed increased ALP activity (4,160 U/L), ALT activity (799 U/L), AST activity (213 U/L), GGT activity (36 U/L), and hyperbilirubinemia (0.8 mg/dL). The owner elected to not pursue any further diagnostic testing.

The genus Bartonella is composed of a group of fastidious, gram-negative bacteria well-adapted to persist intracellularly in humans and animals. Bartonella infection is a well-documented cause of local or diffuse tissue injury in people and infections caused by...
Bartonella spp in human beings have been associated with a wide variety of granulomatous syndromes ranging from classic lymphadenopathy and fever (cat scratch disease) to osteomyelitis, hepatosplenitis, and ocular and thoracic disease.\textsuperscript{1,6-10} In general, the prevalence of granulomatous disease in humans has risen with the increased incidence of AIDS and the more frequent use of immunosuppressive therapy.\textsuperscript{1} There has also been an increase in the number of reports of Bartonella-induced granulomatous hepatitis because of enhanced clinical recognition of this important disease and improved diagnostic capabilities following the isolation and identification of \textit{B henselae} in 1992.\textsuperscript{1} Diseases caused by \textit{Bartonella} spp pose a more serious threat in immunocompromised than in immunocompetent human patients. In particular, disseminated infection with \textit{B quintana} or \textit{B henselae} is characterized by cystic blood-filled spaces affecting solid organs such as the liver (peliosis hepatitis), spleen, and lymph nodes.\textsuperscript{1} In addition to peliosis hepatitis, infection with \textit{B henselae} can cause granulomatous inflammation in the liver and spleen, among other organs.\textsuperscript{1} It has been suggested that \textit{Bartonella} organisms induce granuloma formation when an intact immune system attempts to eliminate the organism, whereas bacillary angiomatosis and peliosis hepatitis most frequently occur in association with defective cell-mediated immunity.\textsuperscript{1}

To date, \textit{Bartonella} spp infection has been implicated as a cause of granulomatous lymphadenitis, granulomatous rhinitis, peliosis hepatitis, and endocarditis in dogs.\textsuperscript{1,12-21} In the present report, we provide data from 2 additional dogs with hepatic disease from which \textit{B henselae} and \textit{B clarridgeiae} DNA was amplified. These findings support the need for prospective diagnostic and therapeutic studies to examine \textit{Bartonella} spp as a potential cause of hepatic injury in dogs.

The cause of granulomatous hepatitis is unknown in as many as a third to a half of human patients,\textsuperscript{2,5} and is frequently undetermined in dogs.\textsuperscript{22} In humans, granulomatous hepatitis secondary to \textit{Bartonella} infection can be associated with diffuse hepatic involvement\textsuperscript{1,12-14} or focal granulomas.\textsuperscript{1,5} The pathogenesis of \textit{Bartonella}-induced granulomatous hepatitis may involve dissemination from regional lymph nodes or via the portal blood system following enteric infection.\textsuperscript{12,26} During the past 15 years, granulomatous hepatitis secondary to \textit{Bartonella} infection has been better recognized by physicians and appears to occur at a higher incidence than previously acknowledged\textsuperscript{12,22} with more than a dozen case reports documenting granulomatous hepatic disease, primarily in children, in the recent literature.\textsuperscript{1,12,13,15,16,22,24}

Although a relatively uncommon diagnosis in dogs, granulomatous hepatitis can be caused by numerous infectious or noninfectious diseases. In a study by Chapman et al,\textsuperscript{12} underlying causes included fungal disease, lymphosarcoma, histiocytosis, intestinal lymphangectasia, and dirofilariasis. However, an underlying cause could not be determined in 1 of 9 dogs.

Clinical syndromes caused by \textit{Bartonella} infection in dogs are still incompletely characterized. Perhaps best characterized is an association between \textit{Bartonella} infection and endocarditis in dogs.\textsuperscript{21} \textit{Bartonella vinsonii (berkhoffii)} DNA has been recovered from the heart valve of a dog with endocarditis,\textsuperscript{21} and \textit{B clarridgeiae} DNA has been recovered from the heart valve and blood of another dog with endocarditis.\textsuperscript{12} \textit{Bartonella} DNA has also been detected in a dog with granulomatous lymphadenitis and in another dog with granulomatous rhinitis.\textsuperscript{12} Although persistent infection with \textit{B vinsonii (berkhoffii)} was detected by means of bacterial culture of serial blood samples obtained over a 13-month period from a healthy dog,\textsuperscript{12} isolation of \textit{Bartonella} spp from the blood of immunocompetent dogs is rare, and to date, no other \textit{Bartonella} spp have been isolated from dogs by means of bacterial culture of blood. A previous report\textsuperscript{25} described peliosis hepatitis in a dog infected with \textit{B henselae}; however, to our knowledge, there are no other reports of the detection of \textit{Bartonella} spp DNA in dogs with other types of hepatic disease. In the present report, PCR testing was requested on the hepatic biopsy specimen from the Basset Hound because of positive western immunoblot results and the reported association between \textit{B henselae} infection and granulomatous hepatitis in people. Previously, we were successful in using 16S rDNA primers to document \textit{B henselae} DNA in a hepatic biopsy specimen from a dog with peliosis hepatitis.\textsuperscript{26} The 16S-23S rDNA primers used for the assays performed on the 2 dogs in the present report were designed to detect and simultaneously differentiate 4 different \textit{Bartonella} spp on the basis of amplicon size. The identity of the amplicon was confirmed as \textit{B henselae} or \textit{B clarridgeiae} in these dogs by means of DNA sequencing. \textit{Bartonella clarridgeiae} DNA was amplified from the Doberman Pinscher's hepatic biopsy specimen fortuitously when a small number of hepatic specimens were processed in an identical manner. Our experience with these cases indicates that additional research is necessary to determine the frequency of \textit{Bartonella} spp infection in dogs with hepatic disease, particularly as the underlying cause is often poorly defined in many dogs with hepatopathy.

A complete necropsy was not performed on the first dog described in the present report, so it was not possible to know with certainty what caused the recurrence of clinical signs. Prior to treatment with azithromycin, granulomatous changes were documented in 2 hepatic biopsy specimens obtained 3 months apart. However, there was no indication of granulomatous inflammation in the postmortem specimen obtained following prolonged antimicrobial treatment. The postmortem histologic changes of fibrosis, bile duct proliferation, and regenerative nodular hyperplasia were considered most consistent with a toxic insult that may have occurred secondary to impaired metabolism of 1 or more of the drugs administered empirically by the owner; alternatively, they may have been associated with an additional underlying cause of this dog's hepatopathy. Unfortunately, prolonged fixation in formalin impaired efforts to extract DNA that could be amplified with the PCR assay. The second dog is still alive 15 months after the initial hepatic biopsy, although the dog's condition has deteriorated, and the dog appears to be in fulminant liver failure. Because
both owners made independent therapeutic decisions, including the protracted use of azithromycin in the Basset Hound and the use of numerous herbal medications in both dogs, it is impossible to assess the therapeutic efficacy of azithromycin in these dogs, and prospective studies are required to evaluate its effectiveness.

The extent to which infection with *Bartonella* spp induces disease in dogs is currently unknown. The unexpected amplification of *B. clarridgeiae* DNA from the liver of a Doberman Pinscher with Doberman hepatopathy further emphasizes the need to investigate a potential role for *Bartonella* spp in the development or perpetuation of chronic liver diseases in dogs. In addition, because of the broad range of clinical syndromes seen in human patients, further studies are warranted to clarify those disease manifestations that can be associated with *Bartonella* infection in dogs. In particular, the role of *Bartonella* spp in the pathogenesis of peliosis hepatis, granulomatous hepatitis, and hepatopathies of undetermined cause should be further investigated. Until these studies have been completed, serologic testing and PCR assaying may be helpful in implicating infection with *Bartonella* spp, so as to facilitate more directed antimicrobial treatment.

References