




STANDARD ARTICLE

Hepatic leptospiral infections in dogs without obvious renal involvement

Katie E. McCallum¹  | Fernando Constantino-Casas¹ | John M. Cullen² |
James H. Warland³ | Harry Swales⁴  | Niamh Lingley⁵ | Andre J. Kortum¹  |
Alex J. Sterritt⁶ | Tristan Cogan⁷ | Penny J. Watson¹

¹Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom

²North Carolina State College of Veterinary Medicine, Raleigh, North Carolina

³Department of Haematology, University of Cambridge, NHS Blood & Transplant Donor Centre, Cambridge, United Kingdom

⁴Small Animal Teaching Hospital, Leahurst Campus, University of Liverpool, Wirral, United Kingdom

⁵Orwell Veterinary Group, Kesgrave, United Kingdom

⁶Highcroft Veterinary Group, Whitchurch Veterinary Centre, Bristol, United Kingdom

⁷University of Bristol, Bristol, United Kingdom

Correspondence

Katie E. McCallum, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, United Kingdom. Email: km664@cam.ac.uk

Background: Reports of chronic hepatitis in dogs caused by *Leptospira* spp. are confined to small case series. Fluorescence in situ hybridization (FISH) allows the identification of spirochetes in liver samples. Consequently, this technique may help elucidate the role of *Leptospira* spp. in cases of chronic hepatitis.

Objectives: To describe cases of hepatic leptospirosis in dogs diagnosed by FISH and subsequent polymerase chain reaction (PCR) speciation, with the absence of clinically relevant renal involvement.

Animals: Ten client-owned dogs.

Methods: Retrospective case series from the University of Cambridge presented between 2013 and 2016 or cases consulted by telephone advice during this time period. Cases were selected based on histopathologically confirmed granulomatous hepatitis and leptospiral organisms identified by FISH and PCR speciation (*Leptospira interrogans/kirschneri*).

Results: All cases had increased liver enzyme activities, and FISH in combination with PCR speciation-confirmed infection with *L. interrogans/kirschneri*. Four dogs underwent repeat liver biopsy, FISH and PCR speciation 4–15 months after initial presentation and doxycycline treatment with 1 dog undergoing repeat sampling at necropsy. Three dogs that underwent repeat biopsy remained positive for *L. interrogans/kirschneri* infection. Six dogs were alive at the time of manuscript preparation and 4 dogs were euthanized as a result of progressive liver disease.

Conclusions and Clinical Importance: The presence of hepatic leptospiral organisms may be associated with chronic granulomatous hepatitis without clinical evidence of renal involvement. Further studies are necessary to elucidate the etiological role of these organisms in the disease.

KEYWORDS

dog, hepatitis, *Leptospira*, United Kingdom

Abbreviations: APTT, activated partial thromboplastin time; CKCS, Cavalier King Charles Spaniel; DGGR, 1-2-o-dilauryl-rac-glycero-3-glutaric acid (6'-methylresorufin) ester; DIC, disseminated intravascular coagulation; FISH, fluorescence in situ hybridization; IgG, immunoglobulin G; MAT, microscopic agglutination test; PCR, polymerase chain reaction; PT, prothrombin time; QVSH, Queen's Veterinary School Hospital; WSAVA, World Small Animal Veterinary Association.

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1 | INTRODUCTION

Leptospirosis is caused by infection with pathogenic gram-negative spirochete bacteria of the genus *Leptospira*.¹ Leptospirosis is maintained in the environment by mammalian reservoir hosts. Clinical disease may result when dogs are exposed to organisms by direct contact with infected urine or indirect transmission by contact with

contaminated water or soil.² The classical presentation of leptospirosis in dogs is acute kidney injury and hepatic failure, pulmonary hemorrhage syndrome, and hemorrhagic diathesis.³ Chronic hepatitis associated with *Leptospira* spp. has been described in infected kennel Foxhounds⁴ and colony Beagles,⁵ and more recently, 2 retrospective studies have described 7/51 and 1/298 dogs with leptospirosis solely affecting the liver, respectively.^{6,7} Previous studies screening for leptospirosis by fluorescence in situ hybridization (FISH)⁸ and polymerase chain reaction (PCR)⁹ in granulomatous hepatitis and chronic hepatitis of dogs, respectively, have been unrewarding. More recently, a case series described the detection of intrahepatic bacteria by FISH in 3 dogs with neutrophilic, pyogranulomatous, or lymphoplasmacytic hepatitis,¹⁰ and FISH has been shown to be useful for the detection of leptospires in liver biopsy specimens in humans.¹¹ The etiological role of leptospiral infection in chronic granulomatous hepatitis in dogs therefore remains obscure.

Chronic hepatitis in dogs has a prevalence of 12% based on post-mortem examination of dogs from a first opinion population in the United Kingdom.¹² Chronic hepatitis is defined by the World Small Animal Veterinary Association (WSAVA) on the basis of histopathological features of hepatocellular necrosis, apoptosis, or both associated with inflammation and concurrent evidence of regeneration and fibrosis.¹³ Granulomatous hepatitis is a type of chronic hepatitis with a macrophage-rich inflammatory infiltrate that may be caused by copper accumulation or have an infectious etiology. Identification of potential underlying causes of chronic hepatitis is essential to enhance our understanding of the pathogenesis and treatment of this condition.

Our aim was to describe the clinical presentation and histopathology of cases of hepatic leptospirosis in dogs without prominent renal involvement as diagnosed by FISH with subsequent PCR speciation of liver histopathology samples.

2 | MATERIALS AND METHODS

Cases were retrospectively selected by reviewing clinical records from the Queen's Veterinary School Hospital (QVSH) from 2013 to 2016 for cases that had FISH performed or cases for which veterinarians had contacted the QVSH for advice. Investigations were performed as part of the normal diagnostic evaluation. Inclusion criteria were cases with a histological diagnosis of granulomatous hepatitis and the presence of leptospires identified by FISH. All cases had a CBC and serum biochemistry performed, and the majority ($n = 9$) had urinalysis performed. Clinical pathology testing was performed at various laboratories. Normal reference ranges for all tests were similar, except for the serum liver enzyme activities, and to adjust for this variable, they were assessed by numerical-fold increase above the upper limit of the normal reference range. Nine cases had leptospiral microscopic agglutination test (MAT) titers, and 4 cases had PCR performed (3 on blood and urine, 1 on urine alone). A positive serological result was based on an antibody titer $\geq 1:800$ against non-vaccinal serovars, $\geq 1:3200$ against vaccinal serovars, or paired serology documenting a 4-fold increase in titer. Cases with clinical evidence of renal involvement: azotemia (serum creatinine concentration >1.58 mg/dL [140 mmol/L]), glucosuria, or both were excluded. In addition, the histopathology database was searched to

identify 2 dogs with granulomatous hepatitis associated with copper storage disease and 2 histologically normal livers. These liver biopsy specimens were subjected to FISH and acted as controls.

2.1 | Liver histopathology

All cases had liver histology, which was reviewed, according to the WSAVA guidelines,¹³ by 2 pathologists (F. Constantino-Casas and J.M. Cullen) who were blinded to the clinical history for these cases. Liver biopsy specimens were stained with hematoxylin and eosin, Sirius red, Warthin-Starry, and rhodanine and underwent semiquantitative copper estimation (minimal [very scattered under $\times 40$ objective]), mild [scattered $\times 40$ objective], moderate [easily seen, $\times 20$ objective], and marked [easily seen, $\times 40$ objective]). Aerobic and anaerobic fresh liver cultures were performed in 6 dogs.

2.2 | FISH methodology

Fluorescence in situ hybridization; was performed on liver samples at the molecular microbiology diagnostics laboratory at Langford Veterinary Services. Formalin-fixed, paraffin-embedded sections first were screened with a 16S ribosomal RNA-directed fluorescent-labeled oligonucleotide probe against all bacteria. The probes were validated against a panel of bacteria to check for cross-reactivity. This procedure was followed by secondary screening with probes specific for other bacterial genera and species (*Leptospira*, *Helicobacter*, *Campylobacter*, *Salmonella*, *Clostridium*, *Escherichia coli*). Images were digitized and thresholds determined based on an unstained control section. Known negative and positive sections for each bacterium were used as controls.

2.3 | PCR speciation

Polymerase chain reaction was carried out using a commercially available test (Langford Veterinary Services, Langford, United Kingdom). This test has a sensitivity of 1.0 and specificity of 0.99 and spans a variable region of the *lipL32* gene,¹⁴ found in pathogenic *Leptospira*. Case DNA was amplified from formalin-fixed paraffin-embedded samples by PCR, and then sequenced to confirm identity as *Leptospira* spp. and single nucleotide polymorphisms used to differentiate species *interrogans* and *kirschneri* from the other pathogenic species by alignment of amplicons against those of known species obtained from Genbank using CLC Sequence Viewer 7 (Qiagen, Denmark). Species *interrogans* and *kirschneri* cannot be distinguished from each other using this method. Both have been reported in the United Kingdom, although *Leptospira kirschneri* is considered uncommon.¹⁵ Tissue from a dog not shown to carry *Leptospira* was used as a negative control alongside a water blank, and each reaction contained an internal control for dog DNA. A synthetic construct amplicon with a single base mismatch to known *Leptospira* spp. was used as a spiked positive control.

3 | RESULTS

Ten dogs were included in the study; their clinical histories are provided in Supporting Information Supplementary Table 1. Age at presentation ranged from 2.8 to 10 years (median, 8.5 years). Breeds

represented included English Springer Spaniel (n = 3), Cocker Spaniel (n = 2), Labrador Retriever (n = 2), and 1 each of Cavalier King Charles Spaniel (CKCS), Jack Russell Terrier, and West Highland White Terrier. Eight dogs were female (7 neutered, 1 intact), and 2 were male (all neutered). Duration of clinical signs ranged from 1 to 16 weeks (median, 1 week). All dogs were fed a commercial manufactured diet. Three dogs were asymptomatic but had persistent increases in serum liver enzyme activities. Serum biochemistry was obtained in 2 of these dogs (cases 1 and 4) as part of preanesthetic screening before a dental procedure and in case 10 during investigation of a hind limb infection.

Clinicopathologic data are shown in Table 1. The CBC documented mild neutrophilia in 2 dogs and thrombocytopenia in 4 dogs. Serum liver enzyme activities were increased in all dogs. Hyperbilirubinemia was identified in 4 dogs. Serum C-reactive protein concentration was measured in 4 dogs and was increased in 1, consistent with systemic inflammation. 1-2-o-dilauryl-rac-glycero-3-glutaric acid (6'-methylresorufin) ester lipase was measured in 6 dogs and was increased in 1 dog. An in-house pancreatic lipase test was performed in one other dog (Idexx SNAP cPL) and was normal. A coagulation profile was performed in 9 dogs and abnormalities were documented in 3 dogs: mild to moderately prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT) in 1 dog, mildly prolonged APTT in 1 dog and moderately prolonged PT and APTT, increased D-dimers and hypocoagulable thromboelastography in 1 dog suggestive of disseminated intravascular coagulation (DIC). A bile acid stimulation test, performed in 4 non-hyperbilirubinemic dogs, identified increased postprandial concentrations suggestive of hepatic dysfunction. Bile cytology and culture was performed in 7 dogs with unremarkable cytology results and negative cultures. Six of these dogs had concurrent liver culture performed, which was negative in 5 dogs, and 1 dog had a sparse growth of *Staphylococcus pseudintermedius* (most likely representing a skin contaminant). Immunoglobulin G concentrations were measured in the CKCS and determined to be low (5.85 mg/mL; reference range, 10-20 mg/mL) consistent with IgG immunodeficiency.¹⁶ Infectious disease testing included toxoplasma serology (n = 1), *Ehrlichia* spp. PCR (n = 1),

Anaplasma spp. PCR (n = 1), *Angiostrongylus vasorum* antigen (n = 2), all of which were negative. Urinalysis was performed in 9 dogs and identified microscopic hematuria in 4 dogs, proteinuria in 2 dogs, and bilirubinuria in 4 dogs. Urine protein:creatinine ratio was determined in 5 dogs with a mild increase in 1 dog (0.78; reference range, 0-0.4). Urine culture was performed in 5 dogs and yielded positive growth of coagulase-positive *Staphylococcus* spp. in 1 dog on a voided sample.

Abdominal ultrasonography was performed in all dogs with abnormalities documented including ascites (n = 2), sedimentation of bile in the gall bladder (n = 3), thickened edematous gall bladder wall (n = 2), microhepatica (n = 3), nodular appearance (n = 3) or patchy echogenicity (n = 7) of hepatic parenchyma, enlarged portal or hepatic veins or both (n = 2), hepatomegaly (n = 1), irregularity of margins of liver lobes (n = 2), and mesenteric lymphadenopathy (n = 2). Abdominocentesis performed in 2 dogs with ascites yielded a fluid that was consistent with a low protein transudate on cytology. Thoracic radiographs and brain magnetic resonance imaging were performed in dog 8 with clinicopathologic changes suggestive of DIC and severe lethargy, hypertension, and bilateral mydriasis, and showed a generalized bronchointerstitial lung pattern and multiple cerebellar and brainstem infarcts, respectively.

Vaccination, serology, PCR, and FISH information are provided in Supporting Information Supplementary Table 1. Nine dogs were vaccinated against leptospirosis, and the vaccination status was unknown in 1 dog. Serology was performed in 9 dogs a median of 4 weeks after presentation (range, 0-20 weeks), with paired serology (4 weeks apart) obtained in 2 dogs. Three of 9 dogs were seropositive: 2 dogs based on a single titer (1:800 to *Australis* and *Bratislava* [non-vaccinal serovars] and 1:3200 to *Copenhageni*, respectively) and 1 dog had a 4-fold increase in convalescent MAT titer to *Copenhageni*. Dog 6 had repeated serology 32 weeks after initial presentation that documented a persistently mildly increased titer of 1/400 to *Copenhageni*; repeated serology at the time of follow-up liver biopsy 15 months after initial presentation was negative.

Leptospira urine PCR performed in 4 dogs at a median of 2.5 weeks (range, 0-66 weeks) after presentation was negative in all

TABLE 1 Selected clinicopathologic variables at presentation

Parameter	Number of dogs			Number of dogs with abnormal value above the reference range	Number of all dogs with abnormal value below the reference range	Reference range
	Number of dogs	Median	Range			
Neutrophils, $\times 10^9/L$	10	8.82	2.75-18.05	2	1	3-11.5
Platelets, $\times 10^3/\mu L$	10	213	31-503	1	4	175-500
PCV, %	10	45	35-58	3	1	37%-55%
Urea, mg/dL (mmol/L)	10	11.9 (4.25)	4.2-17.92 (1.5-6.4)	0	3	7.0-20.72 (2.5-7.4)
Creatinine, mg/dL ($\mu\text{mol/L}$)	10	0.8 (71)	0.49-1.03 (44-91)	0	0	0.38-1.53 (34-136)
Albumin, g/dL (g/L)	10	2.9 (29)	2.2-4.1 (22-41)	0	2	2.5-4.1 (25-41)
Cholesterol, mg/dL (mmol/L)	10	229 (5.95)	57.1-520.5 (1.48-13.48)	3	1	127.41-251.0 (3.3-6.5)
ALT, IU/L	10	9 \times	>1-39 \times	10	0	NA
ALP, IU/L	10	4 \times	>1-14 \times	10	0	NA
Bilirubin, mg/dl ($\mu\text{mol/L}$)	10	1.25 \times	0-8 \times	4	0	NA
Bile acid stimulation test, $\mu\text{mol/L}$ —postprandial	4	111	41-287	4	0	0-22.5
C-reactive protein, mg/L	4	4.3	0.3-10.7	1	0	0-8.8

Abbreviation: ALP, alkaline phosphatase; ALT, alanine aminotransferase.

cases. Blood PCR was positive in 1 of 3 dogs tested. This dog (dog 4) received a 3-week course of doxycycline and subsequently was negative on blood and urine PCR 4 weeks later.

Five dogs had pertinent medical history. Dog 5 had a history of chronic entropion (and secondary *Pseudomonas* spp. conjunctivitis) and folliculitis, and presented to the referring veterinarian with gingivitis. The dog's sibling (dog 6) developed gingivitis 15 months after initial presentation and underwent gingival biopsies and repeat liver biopsy (see below). Dog 3 had a previous diagnosis of hypoadrenocorticism and hypothyroidism before initial presentation. Fifteen months after the first liver biopsy, this dog developed a biliary mucocele and subsequently underwent a cholecystectomy and repeated liver biopsy. Dog 10 had a left hind limb skin infection at initial presentation that was treated with a 5-day course of amoxicillin/clavulanate (Noroclav, Norbrook, United Kingdom) and carprofen (Rimadyl, Zoetis, United Kingdom).

3.1 | Pathology

Liver tissue samples (range, 2-6; median, 4 per case) were obtained laparoscopically (n = 7), during exploratory laparotomy (n = 1), using ultrasound-guided Tru-cut biopsy (Temno Evolution; CareFusion, Vernon Hills, Illinois) (n = 1), and during necropsy (n = 1). Liver biopsies were performed within the first month after initial presentation in all dogs, apart from dog 4 that had increased liver enzyme activities and leptospiremia identified (and was treated with doxycycline) 5 months before the first liver biopsy. Liver biopsy specimens from all dogs had variable amounts of histiocytic, neutrophilic, and lymphocytic cellular infiltration with a portal to random distribution (Figures 1 and 2). These cases were classified as mild (n = 1), mild to moderate (n = 5), and moderate to severe (n = 4) granulomatous hepatitis. In addition, portal fibrosis was moderate to severe in 4 dogs. Repeat liver sampling was performed in 4 dogs. Two dogs with follow-up liver sampling had moderate to severe pyogranulomatous hepatitis that progressed to mild to moderate severity with hepatocyte regenerative hyperplasia and moderate fibrosis (dog 6 was negative on subsequent FISH and PCR speciation). Dog 4 had moderate pyogranulomatous hepatitis on the initial biopsy specimen, and only hepatocyte vacuolar degeneration and a single focus of hepatocyte necrosis on subsequent histopathology. On repeat liver sampling at necropsy, dog 2 showed progression from moderate granulomatous hepatitis at the time of initial histopathology to a mild to moderate hepatitis. Semiquantitative copper scoring was negative in 1 dog, minimal in 3 dogs, and moderate to marked in 3 dogs. Copper accumulation was deemed secondary to the underlying disease process because it was not associated with hepatocyte necrosis and was located periportally. Most cases with moderate to marked copper accumulation had fibrosis, nodular hyperplasia, and moderate to marked inflammatory cellular infiltration. Warthin-Starry staining failed to identify spirochetes in any of the liver sections. Additional screening for infectious etiology was performed using Periodic Acid-Schiff (n = 6), Giemsa (n = 1), Ziehl-Neelsen (n = 2), and Gram (n = 4) staining, which was negative for all dogs tested.

Dog 6 developed gingivitis, lethargy, epistaxis, and peripheral lymphadenopathy and underwent a gingival biopsy procedure 14 months after the original diagnosis of granulomatous hepatitis.

Gingival histopathology disclosed a neutrophilic gingivitis with vasculitis. Culture of the gingiva yielded heavy growth of *Citrobacter freundii* and *Serratia marcescens*. A sibling (dog 5) had gingival biopsies performed by the referring veterinarian that were inconclusive because of their superficial nature (multifocal fibrous, gingival hyperplasia, and osseous metaplasia).

3.2 | FISH results

Fluorescence in situ hybridization; performed on the liver biopsy specimens documented clusters of leptospire in 8 dogs, suggestive of reproducing organisms. Two dogs had evidence of scattered leptospire organisms (Figure 3, Supporting Information Supplemental Table 1). Additionally, concurrent clusters of *Campylobacter coli* and *Salmonella* spp. (n = 1), *C. coli* spp. (n = 1), *Campylobacter jejuni* and *Salmonella* spp. (n = 1), *Salmonella* spp. (n = 1), and *Helicobacter* spp. (n = 1) also were identified, suggestive of the presence of coinfections in these cases. Fluorescence in situ hybridization was performed on the gingival biopsy specimen of dog 6 and disclosed infrequent leptospiral organisms. One additional dog (a 7-year-old male neutered Pomeranian) that underwent liver biopsy and FISH analysis was found through the clinical pathology database search and was diagnosed with severe liver dysplasia and concurrent granulomatous hepatitis, based on necropsy. Fluorescence in situ hybridization results for this dog documented clusters of *Salmonella* spp. and occasional *C. coli* spp. Fluorescence in situ hybridization results for the 4 control dogs (2 dogs with a known cause of granulomatous hepatitis and 2 dogs with histologically normal liver) were negative for leptospiral organisms.

3.3 | PCR and sequencing

Polymerase chain reaction and partial sequencing of *LipL32* in liver (and gingiva of dog 6) samples extracted from tissue blocks identified *L. interrogans/kirschneri*.

3.4 | Treatment

Eight dogs were treated with doxycycline at a dosage of either 5 mg/kg PO q12h or 10 mg/kg PO q24h for a minimum of 14 days (range, 14-21 days). Dog 5 had a diagnosis of leptospirosis at necropsy, and dog 2 was euthanized while on doxycycline treatment. Other treatments administered to the dogs were based on clinician preference and included S-adenosyl methionine (n = 9), potentiated amoxicillin/clavulanate (n = 8), ursodeoxycholic acid (n = 7), spironolactone (n = 4), maropitant (n = 3), metronidazole (n = 3), penicillamine (n = 2), marbofloxacin (n = 2), ranitidine (n = 2), Vitamin K (n = 2), plasma transfusion (n = 2), and enrofloxacin (n = 2).

Follow-up ranged from 22 to 878 days (median, 434 days). Six dogs were alive at the time of manuscript preparation and 4 dogs had been euthanized (3 because of progressive clinical signs of chronic hepatitis and 1 because of liver failure). Three dogs had repeat liver biopsy because of continued increases in liver enzyme activity and 2 of the 3 (dogs 3 and 4) had *Leptospira* spp. identified by FISH. Polymerase chain reaction speciation in these cases confirmed the presence of *L. interrogans/kirschneri*. The dog that did not have leptospire

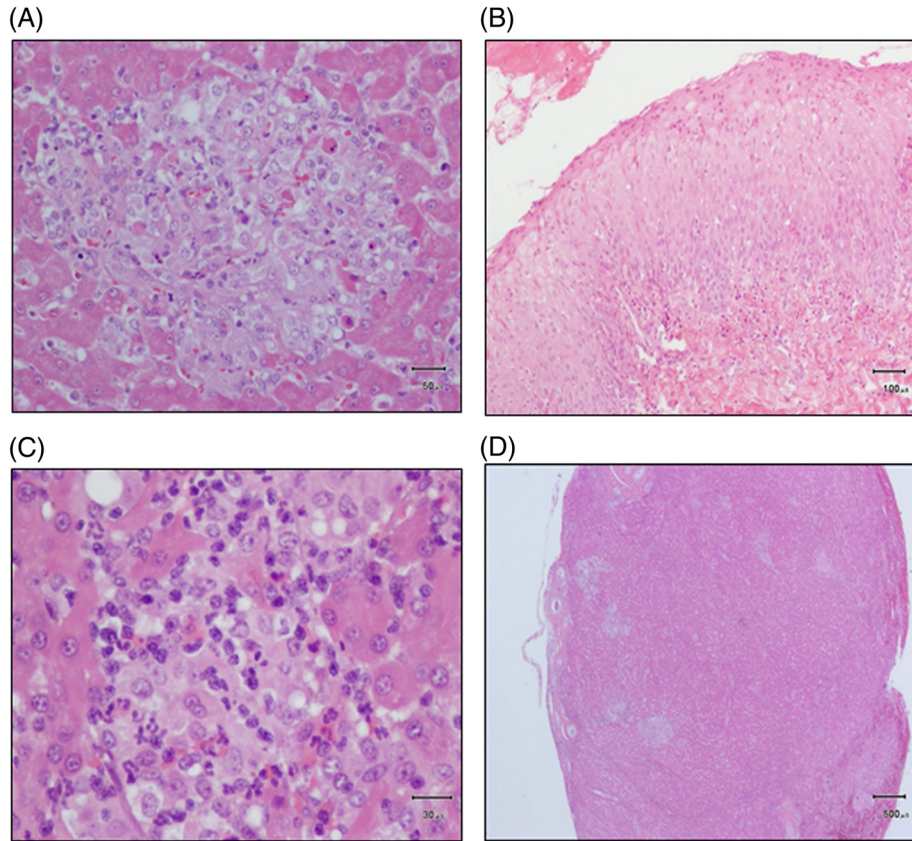


FIGURE 1 A, Original biopsy: Moderate to severe pyogranulomatous inflammation (H&E). B, Gingival biopsy documented neutrophilic gingivitis with vasculitis (H&E). C and D, Repeat liver biopsy documenting mild to moderate pyogranulomatous inflammation with hepatocyte regenerative hyperplasia and moderate fibrosis (H&E). H&E, Hematoxylin and eosin stain

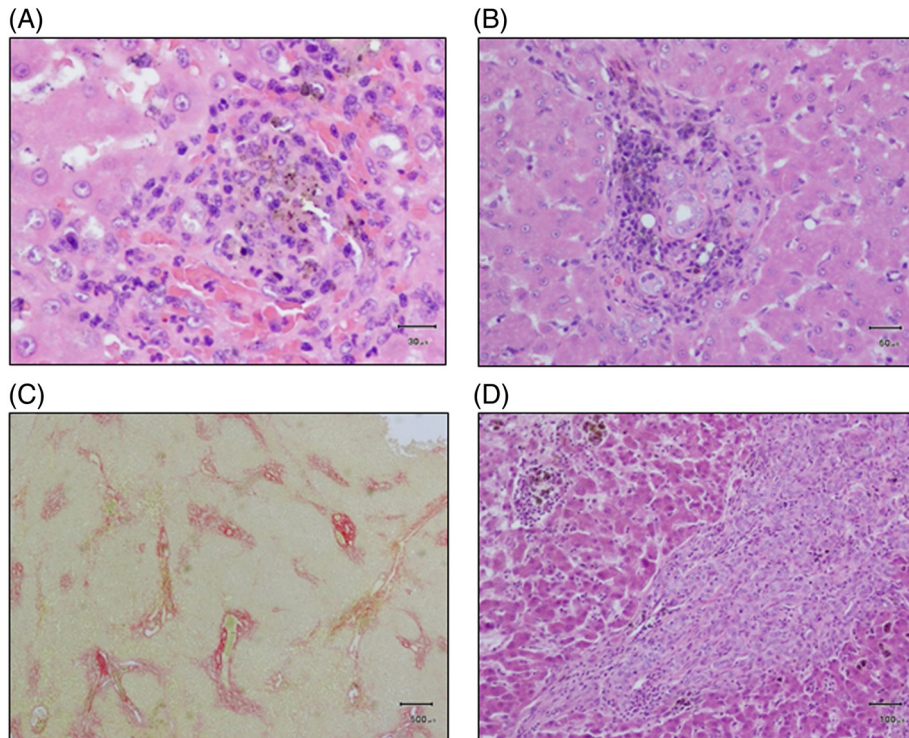


FIGURE 2 A and B, Moderate granulomatous hepatitis with fibrosis and nodular hyperplasia (H&E). C and D, Postmortem liver sample: Granulomatous hepatitis with marked biliary hyperplasia and fibrosis with pigment granuloma formation (Sirius red and H&E, respectively). H&E, Hematoxylin and eosin stain

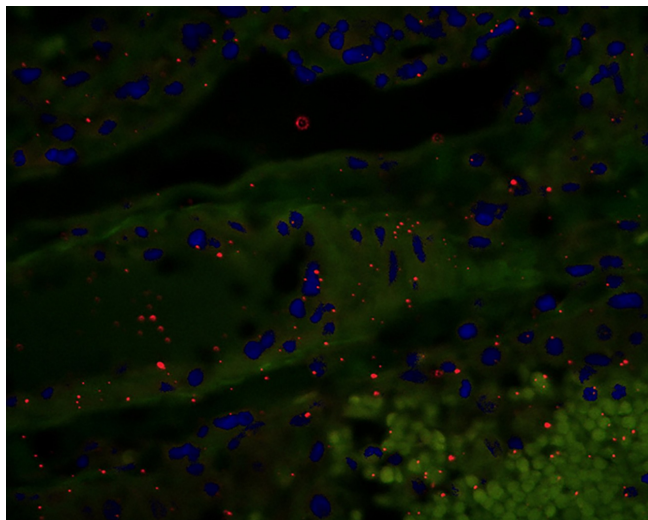


FIGURE 3 FISH image documenting clusters of leptospires (red) within hepatic biopsy specimen

detected in the liver (dog 6) was the one that had the gingival biopsy and 2-week course of doxycycline treatment 1 month before repeat liver biopsy. Interestingly, this dog had evidence of moderate fibrosis on repeat liver biopsy that was not present in the first biopsy specimen. This dog also had clusters of *C. coli* spp. in the initial biopsy specimen that was not present in the second biopsy specimen. The 2 dogs with persistent leptospiral infection were not retreated with doxycycline. However, dog 4 received 11.7 mg/kg amoxicillin/clavulanate PO q12h for 28 days after the second liver biopsy. Dog 2 had a liver sample collected at necropsy and remained positive for *Leptospira* spp. on FISH and PCR speciation. The 6 surviving dogs had follow-up serum biochemistry (range, 158-703 days) that documented persistent increases in liver enzyme activities.

4 | DISCUSSION

This retrospective case series confirms the presence of *Leptospira* spp. organisms by FISH in the livers of dogs with histological evidence of granulomatous hepatitis. In 1 dog, it also identified leptospiral organisms in gingival lesions. The role of *Leptospira* spp. as a primary cause of hepatitis in these dogs remains unclear because persistent or recurrent infection was documented despite doxycycline treatment and because of a lack of a control group to enable us to compare the incidence of leptospiral positivity using FISH. Nonetheless, we documented the presence of *Leptospira* in client-owned dogs with granulomatous hepatitis and also documented *Leptospira*-associated gingivitis.

Clinicopathologic abnormalities in the dogs were sometimes, but not always, typical of previous reports of leptospiral infections. Previously, hepatic leptospiral injury was thought to occur almost exclusively in conjunction with azotemia in dogs.^{17,18} However, 2 case series of leptospirosis associated with chronic hepatitis in dogs in the absence of renal involvement have been published previously, consistent with our findings.^{4,5} Thrombocytopenia (present in 4 dogs in our study) previously has been documented in 6%-58% cases of

leptospirosis and postulated mechanisms include DIC, immune-mediated thrombocytopenia, and platelet aggregation to stimulated vascular endothelium.^{17,19-21} Serum biochemistry in all dogs documented increased liver enzyme activities, although hyperbilirubinemia was not a consistent feature of the disease. Coagulation profile abnormalities were documented in 3/9 dogs with 1 dog having evidence of a bleeding diathesis.

Proteinuria is recognized in leptospiral infections as a consequence of acute interstitial nephritis and tubular dysfunction.²²⁻²⁴ The mild proteinuria seen in 3 of these dogs could reflect renal damage but also could be a nonspecific marker of systemic disease. Glucosuria (a sign of tubular disease documented in dogs with leptospirosis²² was not noted in this population of dogs, thus making clinically relevant renal tubular involvement unlikely.

Although only 3 dogs were considered to have positive serological results based on previously defined criteria, 5 of the dogs that had serology performed had at least 1 titer >1:100. A multi-serogroup agglutinating antibody response is concordant with an active response to natural infection²⁵ and chronically infected animals can have low antibody titers.^{25,26} Previous studies have identified serological responses in 46%-54% of chronic cases.^{4,5} Interpretation of serology requires concurrent assessment of vaccination history. In 1 study, leptospirosis was diagnosed with a single titer of at least 1:800 for 1 or more serogroups,²⁷ but post-vaccinal titers to both vaccinal and non-vaccinal serovars >1:6400 have been observed in noninfected vaccinated dogs.²² Most vaccinated dogs have been shown to become antibody negative by week 15 post-vaccination, although vaccinal titers can persist for 12 months in a small number of dogs,²⁸⁻³⁰ thus complicating the diagnosis of leptospirosis in vaccinated dogs. Leptospirosis previously has been identified in a dog in the absence of detectable antibody response.³¹ The lack of consistent serological response in our study could be a result of sequestration of leptospires, localized infections, or infection with less pathogenic strains.^{5,25,32}

Two of 3 dogs with positive serology in our study had significant MAT titers to serovar *Copenhageni*, which belongs to the *Icterohaemorrhagiae* serogroup. Furthermore, 4 dogs had evidence of titers \geq 1:100 to serovar *Copenhageni*. Although a positive association between infection with serogroup *Icterohaemorrhagiae* and disease limited to the liver has been reported previously,⁶ another study failed to show an association between the infecting serovar and the pathophysiology of leptospirosis.³³

Urine and blood PCR for leptospires was performed in 4 dogs and was negative in all but 1 of the dogs that had a positive blood PCR. Leptospires circulate transiently for the first 10 days after infection and thereafter appear in urine,^{34,35} and therefore the timing of sampling is important. The dog with leptospiremia was treated with doxycycline, and PCR results 4 weeks later were negative. This finding may have reflected the acute phase of infection (although no serology results were available from this time to document exposure). PCR is not affected by vaccination status.²⁷ A negative PCR result does not rule out leptospirosis and, in fact, is not necessarily unexpected, given the suspected chronic nature of the infection, prior antibiotic treatment, and lack of renal involvement. Furthermore, false-negative results can occur because of intermittent shedding.³⁶ All 3 dogs with

negative PCR results had received antibiotics, whereas the dog with a positive PCR had not received any antibiotic treatment.

Hepatic histopathological features previously described in cases of chronic hepatitis because of leptospirosis include mild to marked inflammatory cellular infiltration (usually portal and centrilobular) characterized predominantly by lymphocytes with some neutrophils and occasional plasma cells and macrophages. Interestingly, cholangiolar proliferation, hepatocellular vacuolization, bile stasis, and hemosiderin granulomas were noted, similar to our findings.^{4,5} Where a second liver sample was examined, the severity of the inflammatory infiltrate was less pronounced, which might indicate recovery from *Leptospira* infection, but further investigation is needed to evaluate this hypothesis, given the persistence of leptospiral organisms in the second liver biopsy specimen and the variable time frame from the first to second liver biopsy. Spirochete bacteria were not identified in any of the liver samples using Warthin-Starry staining. However, silver staining to directly visualize spirochaetes is known to be insensitive for the detection of leptospires³⁶ and may be negative if few organisms are present.³⁷

Documented causes of granulomatous hepatitis in dogs include bacterial (*Mycobacterium* spp., *Bartonella* spp., *Nocardia* spp., *Actinomyces* spp., *Rhodococcus* spp., *Helicobacter canis*), fungal (*Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatidis*), protozoan (*Leishmania infantum*, *Toxoplasma gondii*, *Neospora canis*), and metazoan parasites (visceral larval migrans, schistosomiasis) and copper accumulation.^{8,38} Additional staining of the liver biopsy specimens for infectious organisms using Periodic Acid-Schiff, Gram staining, and Ziehl-Neelsen staining were not deemed necessary in all cases by the pathologist assessing the sample, but may have been useful to exclude other causes of granulomatous hepatitis. However, staining was negative in all cases that were tested and the eubacterial probe used for FISH would have identified any other clinically relevant bacterial presence.

Leptospirosis was diagnosed by a combination of FISH and subsequent PCR speciation in all cases. Fluorescence in situ hybridization previously has been used to diagnose leptospirosis in a dog.³⁹ Fluorescence in situ hybridization on tissue samples is considered sensitive and specific for the organism against which the FISH probe is targeted. Further studies assessing FISH in granulomatous hepatitis, normal liver, and other hepatic diseases of dogs are warranted to assess the role of leptospirosis in these cases. Polymerase chain reaction on liver tissue previously has been found to be useful in detecting chronic forms of leptospirosis.⁵

Fluorescence in situ hybridization identified concurrent infections with *Salmonella*, *Campylobacter*, and *Helicobacter* spp. in 5 dogs. Although *Salmonella* and *Campylobacter* infections previously have not been recognized as a cause of granulomatous hepatitis, *H. canis* has been associated with multifocal necrotizing hepatitis in a dog.⁴⁰ *Salmonella* also has been associated with acute hepatic necrosis⁴¹ and was diagnosed by FISH in a dog that underwent liver biopsy at the authors' institution and subsequently was diagnosed with granulomatous hepatitis and liver dysplasia. Increased prevalence of enteropathogenic infections can be seen with the feeding of raw food diets,⁴² and these organisms may gain access to the liver via the portal circulation. There was however no history of any dog being fed a raw

diet in this case series. Leptospirosis serology was supportive of infection in only 1 dog in which concurrent bacteria were identified by FISH. One of the dogs with coinfection by *C. coli* had a repeat liver biopsy that documented no evidence of persistent *Campylobacter* infection, despite the lack of specific treatment for *Campylobacter* (ie, macrolides or fluoroquinolones). It would have been useful to perform fecal cultures in the dogs infected with *Salmonella* and *Campylobacter*, and a limitation of this study was the lack of treatment for these organisms or subsequent investigations to confirm whether these infections had been eradicated.

Interestingly, persistent leptospiral infection was documented in 3 of 4 dogs that had repeat liver biopsies using FISH and PCR speciation to investigate persistently increased serum liver enzyme activities. In all 3 dogs, serum liver enzyme activity decreased after the second liver biopsy, and all were still alive at the time of manuscript preparation. The cause of these persistent infections is unknown. They could represent reinfection after exposure to the same environmental source, lack of drug penetration to the site of infection in the liver, insufficient duration of treatment, or antimicrobial-resistant infections. However, resistance to doxycycline is thought to be rare in leptospirosis in dogs.^{3,43} All dogs were treated for a minimum of 14 days with doxycycline, which is the recommended treatment for dogs with leptospirosis.^{3,22,36}

An interesting feature was the involvement of 2 siblings with confirmed leptospirosis, both of which had gingivitis before and during the course of their disease. Although FISH was not performed on the gingiva of the euthanized dog, FISH and PCR speciation on the gingival lesions of dog 6 identified scattered *L. interrogans/kirschneri*. Based on the lack of clustering, it was unclear whether these organisms were alive and dividing or dead. Interestingly, this dog had a repeat liver biopsy, and no leptospiral organisms were identified by FISH or PCR speciation, suggestive of clearance of infection. Serology was also negative at the time of the second liver biopsy, and this dog previously had 2 titers against *L. copenhageni* of 1:400. This dog had recurrence of increased serum liver enzyme activities and lymphadenopathy 23 months after initial presentation and was diagnosed with granulomatous lymphadenitis, which responded to treatment with 1 mg/kg/d of prednisolone. The gingiva is a secondary lymphoid organ, and the presence of a few leptospires in this region could also reflect the normal immune response to the organisms (ie, leptospiral DNA being carried by the lymphoid system to the gingiva). However, the gingiva could represent a portal of entry and source of reinfection from an environmental source. Interestingly, mucosal surfaces are an important portal of entry for leptospires in humans.⁴⁴ Furthermore, a previous case of leptospirosis in a young adult person has been attributed to infection by the cutaneous route.⁴⁵ A surface protein (OmpL37) has been shown to be expressed by *Leptospira* pathogenic for humans during infection, which allows leptospiral organisms to bind to skin and vascular elastin.⁴⁶ More studies of potential *Leptospira* involvement in skin and gingival lesions in dogs are warranted.

Nine of the dogs in our study were vaccinated against leptospirosis, which highlights the possibility of infection with a serovar that is not present in currently available vaccines. The major serogroups which dogs in Europe seroconvert to are *Leptospira* serogroups *Grip-potyphosa* (species *kirschneri*), *Icterohaemorrhagiae*, *Australis*, *Sejroe*,

and *canicola* (all species *interrogans*).¹⁵ This observation has led to the development of quadrivalent vaccines that include protection against serogroups *grippityphosa* and *australis*, as well as *canicola* and *icterohaemorrhagiae*. Vaccination has been shown to provide protective immunity after extreme challenge for 12 months with L4 vaccination⁴⁷ and 14 months with L2 vaccination.⁴⁸ However, vaccination may not prevent infection, especially if dogs are exposed to a high infectious challenge or highly invasive strain.^{49,50}

The survival rate in our study was 60% (6/10 dogs). Previous survival rates documented in the veterinary literature range from 52% to 83%.^{7,18,33,51,52} The dogs that did not survive generally were younger (median, 3.5 years compared to the overall median, 8.5 years), but the numbers were too low to assess statistically. All 4 non-survivors presented with clinical signs. Two of the 4 dogs had signs of hepatic dysfunction (eg, hypoalbuminemia, hyperbilirubinemia, ascites) and the other 2 had coinfections with *Salmonella* and *Campylobacter*. The inflammation was classified as mild to moderate in 1 dog and moderate to severe in 3 dogs, with evidence of moderate to severe fibrosis in 3 dogs. Of the 6 surviving dogs, 3 remained asymptomatic, none had signs of hepatic dysfunction, 3 had coinfections with *C. coli*, *Salmonella* and *Helicobacter* spp., and all 6 had decreased serum liver enzyme activities on follow-up serum biochemistry compared to initial presentation. No association between duration of clinical signs and outcome was observed (the majority of dogs experienced 1-2 weeks of clinical signs), but all 3 asymptomatic dogs remained asymptomatic and still were alive at the time of follow-up.

This case series presents a possible association between chronic granulomatous hepatitis and leptospirosis in dogs. These atypical infections simply may have been diagnosed as a result of advances in available diagnostic techniques (FISH/PCR), an increase in the prevalence of these infections, or both. We were unable to identify a seasonal pattern on the basis of the month of presentation because of the low number of cases, and it is difficult to ascertain the exact time of infection in these chronic infections. Underlying immunosuppression could explain the presence of leptospiral organisms in some of the dogs: dog 3 had concurrent hypothyroidism and hypoadrenocorticism, dog 1 was diagnosed with IgG deficiency, which previously has been shown to predispose to *Pneumocystis carinii* infection,¹⁶ and 50% of the dogs had coinfections detected by FISH, which may reflect an increased risk of developing infections. Experimental studies have shown that both leptospiral virulence and host immune response account for the severity and clinical features of the disease.^{53,54} The role of the immune system in the development of chronic leptospirosis in dogs therefore warrants further investigation.

The limitations of this study include its retrospective nature, including some clinical records being incomplete and lack of standardization of clinical management. Prospective paired leptospiral serology and PCR results were not available for all the dogs. Paired samples can increase the sensitivity of serology for the diagnosis of leptospirosis from 50% to 100%.²⁷ Furthermore, the timing of these tests was variable in relation to the initial presentation of the dogs. Renal histopathology was not available, and serum creatinine concentration is an insensitive marker of glomerular filtration rate. Therefore, it is possible that some of these dogs had concurrent but subclinical renal involvement. Furthermore, 1 dog did not have a urinalysis performed. Polymerase chain reaction could not differentiate infection with *L. interrogans* from that with

L. kirschneri, but it is important to note that there was no serological response to serovar *grippityphosa* in this cohort of dogs. Limited information is available on the prevalence of leptospiral organisms detected by FISH in healthy dogs.

Future research should include performing paired serology and both blood and urine PCR in all suspected cases. Furthermore, potential environmental sources of infection should be investigated, especially those with persistent or recurrent liver involvement. Further investigation of the immune status of affected dogs would be helpful to assess whether immunosuppression is contributing to the pathogenesis. Polymerase chain reaction identification of the infecting strain is being utilized in human medicine^{55,56} and may aid in studying the epidemiology of this disease. The prevalence of leptospires in tissues of healthy dogs also should be assessed by FISH.

In conclusion, leptospirosis should be considered as a potential cause of granulomatous hepatitis, even in vaccinated dogs and dogs without evidence of renal dysfunction or seroconversion. Currently, it is unclear whether identified leptospires are the primary cause of hepatitis, occur secondary to chronic hepatitis, or are a trigger for chronic immune-mediated liver disease.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

One case received a 2-week course of 5 mg/kg PO SID azithromycin (Zithromax, Pfizer, UK).

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Katie E. McCallum  <https://orcid.org/0000-0001-6153-0687>

Harry Swales  <https://orcid.org/0000-0002-9341-1882>

Andre J. Kortum  <https://orcid.org/0000-0002-9760-733X>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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