Retrospective Immunohistochemical Detection of Leptospira in Dogs with Renal Pathology

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ABSTRACT

The purpose of this study was to retrospectively evaluate renal tissue from dogs with renal pathology for evidence of leptospira. The pathology database at Tufts University Cummings School of Veterinary Medicine, which included accessions from Tufts Foster Hospital for Small Animals as well as a number of private small animal practices, was searched for dogs that had renal pathology of any type recorded as one of the diagnoses from January 1999 through July 2002. Sections of renal tissue from 265 cases underwent immunohistochemistry staining for leptospira. For each case, an attempt was made to obtain the following data: signalment, state of origin, BUN, serum creatinine, alkaline phosphatase, alanine aminotransferase, and bilirubin concentration, titers for leptospirosis, and vaccination status. Of the 265 cases, 48 (18.1%) were positive for the presence of leptospiral antigen. There was no correlation between gender, breed, state of origin, histopathologic diagnosis, presence of azotemia, or current vaccination for leptospirosis and a positive test.

INTRODUCTION

The incidence of clinical leptospirosis in dogs in North America has been increasing over the past two decades.[1,2] These infections have been diagnosed primarily by serology, and have been most commonly due to *Leptospira kirschneri* serovar Grippotyphosa and *L. interrogans* serovar Pomona.[3-9] A smaller number have been attributed to *L. interrogans* serovars Bratislava[10] and Autumnalis.[11] The predominant clinical presentation has been acute renal failure, in contrast to older reports in which both the liver and kidney were affected.

Evaluation of dogs for infection with leptospirosis can be done in several ways. Bacterial culture is the gold standard for

diagnosis. However, culture of leptospires is technically difficult, and prior administration of antibiotics may affect bacterial growth.[12] Fluorescent antibody testing can detect leptospires in urine, blood, or tissue, but requires unfixed tissue.[13] Serology can provide evidence of infection, although such infection does not necessarily produce clinical signs. Immunohistochemistry has been shown to be a valid technique for detection of leptospires, and has the advantage of being able to be performed on formalin-fixed tissue.[14]

Subclinical infections with leptospirosis in dogs in the U.S. may be more common than is recognized. A 2007 study found that 309 of 1,241 (24.9%) healthy dogs from Michigan had antibody titers against at least one of the six *Leptospira* serovars, which suggested exposure to *Leptospira* spp. Prevalence of antibodies was highest to serovar Grippotyphosa, followed by Bratislava, Canicola, Icterohaemorrhagiae, and Pomona.[15] Another group of healthy dogs in Washington State reveaked that 27/158 (17.1%) had an antibody titer greater than or equal to 1:100) to any serovar, with the most frequently detected serovars being Autumnalis, Icterohemorrhagiae, and Canicola.[16] A study comparing polymerase chain reaction (PCR) testing, culture, and serology found that the urine of 41 of 500 (8.2%) dogs had positive PCR results, although only three dogs were clinically ill.[17] Renal disease and renal failure are common causes of morbidity and mortality in older dogs.[18] The cause is rarely identified, and therefore, specific recommendations for treatment or prevention are not possible. Historically, mild or subclinical infections with leptospirosis were considered to be causes of ongoing renal damage and renal failure in the older dog.[19-21] However, this theory fell out of favor because studies on the role of leptospirosis in producing long-term renal damage produced conflicting results.[19,20-23]

The increasing incidence of clinical leptospirosis in dogs, and serologic data suggesting subclinical infection in many dogs, suggest that the association of leptospirosis and chronic renal disease warrants further investigation. The purpose of this study was to retrospectively examine renal tissue from dogs with renal pathology for the presence of leptospires.

MATERIALS AND METHODS

Study design

The pathology database at Tufts University Cummings School of Veterinary Medicine (TCSVM) was searched for dogs that had renal pathology of any type recorded as one of the diagnoses from January 1999 through July 2002. This database consisted of cases accessioned through the Tufts Diagnostic Laboratory through 1999 and the Tufts Section of Pathology – Department of Biomedical Sciences for 2000-2002. The Tufts Diagnostic Laboratory accepted accessions from the Tufts Foster Hospital for Small Animals as well as from outside private practices, the majority of which were in New England. The laboratory changed ownership at the end of 1999, and cases in the database after that date were all from the Tufts Foster Hospital for Small Animals. Cases were included whether the tissue was obtained ante-mortem as a diagnostic biopsy, or from post-mortem examination. Cases were selected in chronological order, and consisted of biopsy samples from 1999 and necropsies from 1999-2002. Sections of kidney tissue from each case were cut and mounted for immunohistochemistry testing for the presence of leptospiral antigens.

For each case, an attempt was made to locate the medical record and examine it if the dog was a patient at Tufts, or to obtain information about the patient from the private practice if the sample had been submitted through the diagnostic laboratory then operated by TCSVM. In many of the cases from private practices, data could not be obtained because the medical record had been discarded after the dog died. Data collected, as available, included signalment, BUN, serum creatinine, alkaline phosphatase, alanine aminotransferase, and bilirubin concentration, titers for leptospirosis, and

vaccination status. Titers ≥1:800 were considered positive. Dogs were considered to be currently vaccinated for leptospirosis if the last vaccine had been given within 12 months of the time renal histopathology was performed.

Because most of the dogs lived in New England, breeds of dogs seen at Tufts Foster Hospital for Small Animals (which handles referral cases from approximately the same geographic area as the accessions) for the years 2000-2002 were used as the reference population. Dogs recorded as a breed crossed with another breed (eg, Labrador/Shepherd cross) were counted as the first breed listed.

Immunohistochemistry

Slide sections were incubated in antigen retrieval buffera in a preheated steamer and heated for 20 min at 100 C. Slides were removed from the steamer and left in the retrieval solution at room temperature to cool down for 20 min, rinsed in distilled water, and transferred to Tris-HCl buffer. Endogenous peroxidase was blocked for 15 minutes with 3% hydrogen peroxide. Nonspecific immunoglobulin binding blocking was done by a 10 minute incubation with a protein-blocking agentb prior to application of the primary antibody. A rabbit-polyclonal anti-L. interrogans serovar Copenhageni that cross-reacts with other leptospiral serovars, was used as the primary antibody at a concentration of 1:500 Sections were stained with an autostainer using a labeled streptavidin-immunoperoxidase staining procedure.c The immunoreaction was visualized with AEC.d Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted. Sections of kidney and liver from dogs experimentally infected with serovar Grippotyphosa served as positive controls. The positive control tissues were culture positive for serovar grippotyphosa and leptospires were also detected by immunofluorescence testing. For negative controls the primary antibody was replaced with Tris-HCl buffer. Because the antibody used is cross-reactive with many leptospiral serovars, it is not possible to determine the specific serovars present in tissues using this technique.

Sections were read by one pathologist (Kiupel) and positive slides were confirmed by another pathologist (Bolin). Immunohistochemistry was considered positive if there was strong staining of organisms or fragments of organisms, negative if no staining was observed, and suspect if there was strong staining of the cytoplasm of cells without evidence of organisms. For purposes of data analysis, samples classified as suspect were considered to be negative.

Statistical Analysis

Statistical analysis was performed with the SPSS statistical computer program. Descriptive data is presented as mean with standard deviation. Comparison of breeds of dogs with renal pathology to breeds of dogs seen at Tufts Foster Hospital for Small Animals was done using the Z test for 2 proportions. Comparisons of gender, breed, state of origin, current vaccination for leptospirosis, titers for leptospirosis, and histopathologic diagnosis to immunohistochemistry staining for the presence of leptospires were done using Chi-squared analysis, or Fisher's exact test when the expected frequency in any cell was <5. Statistical significance was set at $P < .05$.

RESULTS

A total of 281 cases of dogs with renal pathology were identified. Of these, 265 cases had sufficient kidney tissue remaining in the paraffin block for immunohistochemical staining. There were 42 (15.8%) intact male dogs, 97 (36.6%) castrated males, 20 (7.5%) intact females, and 104 (39.2%) spayed females. Gender was not recorded for 2 (0.8%) of the dogs (Table 1). The mean age was 7.7 ± 3.7 years. A wide variety of breeds were represented. None of the 10 most common breeds in this study had an increased risk of renal pathology compared to breed prevalence in the reference population (Table 2).

The majority of dogs resided in New England (Table 3). Vaccination status for

Table 1. *Gender of Dogs*

GENDER	NUMBER OF DOGS	NUMBER POSITIVE FOR-LEPTOSPIROSIS
FEMALE	20	1
SPAYED FEMALE	104	16
MALE	42	13
CASTRATED MALE	91	17
UNKNOWN	2	1

Table 2. *Most Common Breeds of Dogs with Renal Pathology*

BREED	NUMBER OF DOGS	NUMBER POSITIVE FOR-LEPTOSPIROSIS
Labrador Retriever	40	9
Golden Retriever	29	5
German Shepherd Dog	18	2
Rottweiler	13	1
Cocker Spaniel	11	2
Mixed breed	9	3
Beagle	5	2
Doberman	4	1
Boxer	4	1
Dachshund	3	1

**Dogs recorded as a breed crossed with another breed were considered to be of the first breed listed; e.g., Labrador/ Shepherd cross was counted as a Labrador Retriever*

Table 3. *Geographic Distribution of Cases*

State or Country	Number of Cases	Number positive for Leplospirosis
Massachusetts	182	35
New Hampshire	21	5
New Jersey	18	0
Connecticut	15	3
Rhode Island	10	2
Maine	6	1
New York	5	1
Vermont	2	0
California	2	0
Hong Kong	2	1
Michigan	1	0
Virginia	1	0

leptospirosis was known for 128 (48%) of the dogs. Of these, 101 were currently vaccinated and 27 were not. It was not possible from the available records or information from private practices to determine the leptospiral serovars for which dogs had been vaccinated.

The types of renal pathology included inflammatory, degenerative, and neoplastic lesions. Because the kidneys of many dogs had more than one type of lesion, the diagnosis listed first on the pathology report was considered to be the predominant abnormality (Table 4). For statistical analysis, the diagnoses were further grouped into five categories. Category 1 included dogs that had glomerulonephritis as the predominant lesion; 2 - chronic interstitial nephritis; 3 - acute tubular necrosis; 4 - acute interstitial nephritis and 5 - other (included diagnoses of neoplasia, amyloidosis, congestion, hemorrhage, infarcts, pyelonephritis, renal dysplasia, and immature glomeruli). One-half (134) of the dogs had lesions classified as glomerulonephritis, and 87% of these samples were from necropsies. One hundred dogs had normal renal function (serum creatinine concentration <2.5 mg/dl) and 54 were azotemic (serum creatinine < 2.5 mg/dl). Serum creatinine concentrations were not available for 111 dogs. There was no correlation between azotemia and the type of renal pathology. It could not be determined from available information whether the dogs were showing clinical signs related to the renal pathology or azotemia.

Titers for leptospirosis were performed on 32 dogs, of which 4 were positive. All four dogs were azotemic. Two dogs had highest titers to serovar Pomona, and two to Bratislava. None had a positive titer to serovar Canicola. None of the four had a positive histochemistry test, although one had a "suspicious" result that was classified as negative for data analysis. Three of the dogs had renal lesions consistent with chronic renal disease (membranoproliferative glomerulonephritis with mineralization or fibrosis).

Of the 265 kidney samples tested, 48 (18.1%) were positive for the presence of leptospiral antigen based upon immunohistochemistry (43 from necropsy samples, 2 from biopsies, and 1 from a sample of unknown source). An additional 22 cases were classified as "suspect" for leptospiral antigen. These were considered negative for purposes of statistics. None of the 10 most common breeds had an increased risk of having a positive immunohistochemistry test for leptospirosis compared to the reference population (Table 1). In addition, there was no correlation between gender (Table 1), breed (Table 2), state of origin (Table 3), histopathologic diagnosis (Table 4), presence of azotemia (Table 5), or current vaccination for leptospirosis (Table 6), and a positive immunohistochemistry test for leptospirosis. Although there was no correlation with gender, the number of male dogs with a positive test approached significance (Table 1) ($P < .066$).

DISCUSSION

The results of this study showed that 18% of the dogs with renal pathology diagnosed by the section of pathology at Tufts University Cummings School of Veterinary Medicine between 1999 and 2002 and evaluated in this study were infected with *Leptospira*. These results are consistent with serologic surveys published periodically over the past 50 years.[5,14,15,24-26] Because subclinical leptospiral infections appear to be common, these dogs would not necessarily have shown typical clinical signs of leptospirosis. The leptospiral antigen can persist for some time, resulting in a positive immunohistochemistry result.

In the mid-1900's, leptospirosis in dogs was investigated to determine whether it could be responsible for chronic renal disease. In one study, dogs infected with leptospiral serovars Canicola or Icterohaemorrhagiae showed no difference from control dogs in glomerular filtration rate (GFR), renal plasma flow (RPF), or histopathology up to 1,570 days after infection.[22] Other au-

Table 4. *Classification of Renal Pathology**

Diagnosis	Number of Cases	Number positive for Leplospirosis
Glomerulonephritis	134	26
Other**	75	9
Chronic interstitial nephritis	37	8
Acute tubular necrosis	14	4
Acute interstitial nephritis	5	1

**For cases that had more than one type of renal pathology, the lesion listed first in the pathology report was counted as the diagnosis.*
***Includes neoplasia, amyloidosis, congestion, hemorrhage, infarcts, pyelonephritis, renal dysplasia, and immature glomeruli*

Table 5. *Presence of Azotemia*

Azotemic*	Number of Dogs	Number positive for Leplospirosis
Yes	54	12
No	100	16
Unknown	111	20

Table 6. *Leptospiral Vaccination Status*

Currently Vaccinated	Number of Dogs	Number positive for Leplospirosis
Yes	101	16
No	27	6
Unknown	137	26

**Dog were considered currently vaccinated if the last vaccine for leptospirosis had been given within 12 months of the time renal histopathology was performed.*

thors questioned the role of leptospirosis in dogs with chronic interstitial nephritis based upon inability to "follow" progression of histopathologic lesions from the subacute to the chronic stage,[21] lack of serum antibody titers, or the inability to demonstrate leptospires on histopathology. In contrast, some investigators believed that the renal lesions that were seen in dogs with leptospirosis (including those reported in previous studies[21]) could progress and result in chronic interstitial nephritis and renal failure.[19,20,23] Additional support for a role for leptospirosis was provided by serologic surveys of dogs for evidence of leptospirosis. These indicated a relatively high incidence of infection in various states in the U.S. (9% to 30%).[5,24-27]

Immunohistochemistry has been shown to be valuable in identifying leptospires and leptospiral antigens in tissues from animals and humans[28-32] and is the only technique that can be routinely applied in formalin-fixed materials. However, using this procedure it is not possible to determine the serovars of the organisms detected in the kidney tissue as the antibody used is cross-reactive with multiple leptospiral serovars. Serum antibody titers of the infected dogs would likely provide information regarding the infecting leptospiral serovars but titers were not available for most dogs in this study.

No correlation was found in this study between vaccination for leptospirosis and

a positive immunohistochemistry test. All leptospiral vaccines contain inactivated whole or subunits of leptospiral organisms. Leptospiral vaccines are administered subcutaneously, and most of the antigen remains in local tissue. Immediately after vaccination, it might be possible that lymph nodes draining the vaccination site might contain some vaccine antigen. However, it would not be expected that killed vaccine antigen would be found in the kidney.

Serum antibodies to leptospiral antigens increase after vaccination and after natural exposure or infection. Low titers (less than 1:200) are associated with previous infection or vaccination, although high titers are occasionally observed soon after vaccination.[12] The commonly accepted criteria for clinical diagnosis of leptospirosis is a single titer greater than 1:800 in conjunction with appropriate clinical signs, or a four-fold rise in titer over a 2- to 4-week interval.[10,12] Because the data in these cases was collected at one point in time, a titer of 1:800 was considered positive.

Four dogs with positive titers for leptospirosis had negative results on immunohistochemistry staining. All of these dogs were azotemic, and infection with leptospirosis was suspected antemortem. Two of the dogs had a histologic diagnosis of chronic membranoproliferative glomerulonephritis with interstitial fibrosis and/or mineralization of Bowman's capsule. One of these dogs had titers of 1:1600 to *L*. Pomona and Bratislava, and the other titers of 1:3200 to *L*. Pomona and 1:1600 to *L*. Bratislava. One had mild to moderate interstitial nephritis and mild tubular necrosis, and titers of 1:3200 to *L*. Pomona and 1:6400 to *L*. Bratislava. The fourth dog had bilateral renal arteriolar infarction with mild to moderate glomerulonephritis and mild interstitial nephritis, and titers of 1:6400 to *L*. Bratislava and 1:800 to *L*. Grippotyphosa. This last dog had a suspicious result on immunohistochemistry that may have indicated the presence of leptospires, but because of the classification protocol, was considered negative.

Immunohistochemistry has been shown to have similar sensitivity and specificity as silver staining of renal tissue for leptospires.[31] It is possible that these dogs had insufficient leptospiral antigen in renal cells to result in a positive stain, or that antigen was not present in the small section of kidney tissue that was tested. All of these dogs were treated with leptospirocidal antibiotics for 2 to 7 days prior to death or euthanasia, which may have eliminated or significantly reduced the number of organisms from the kidneys. Immunohistochemical testing of animals with leptospirosis that have received antibiotic therapy has not been reported. Alternatively, these dogs may have been infected with leptospirosis some time in the past, and while the organism had been cleared from the kidney, antibody titers persisted.

Because this survey included only dogs with renal pathology, it is not possible to draw any conclusion about the contribution of leptospires to the renal lesions. However, nine dogs tested positive that had only neoplastic or non-specific lesions (such as congestion) in the kidneys, without significant inflammatory or degenerative changes. This might suggest that leptospires can, at least in some cases, infect the kidney without causing pathology or clinical disease. It is not possible to know how long these dogs had been infected, and whether lesions might have developed if the dogs had not succumbed to another disease. Based upon the results of the current study, further investigation and comparisons of the presence of leptospires in renal tissue from dogs with and without pathology are indicated.

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