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Evaluation by polymerase chain reaction assay of persistent shedding of pathogenic leptospires in the urine of dogs with leptospirosis

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Abstract

Background: Persistent leptospiruria in naturally infected dogs occurs despite appropriate antibiotic treatment.

Hypothesis/Objectives: To determine the frequency of persistent leptospiruria in naturally infected dogs and the association of persistent leptospiruria with different antibiotic treatments.

Animals: Thirty-two dogs of varying age and breed diagnosed with leptospirosis via urine polymerase chain reaction assay (PCR).

Methods: A prospective observational study of dogs diagnosed with leptospirosis was undertaken to determine the frequency of persistent leptospiruria as determined by PCR. Clinical presentation of leptospirosis, antibiotic treatment, serum creatinine concentration, and outcome were recorded.

Results: Fifteen of 32 dogs had a negative urine PCR on the first submission in the study, 5 of 15 received only an aminopenicillin. The remaining 17 dogs had a negative urine PCR on the second (n = 6 dogs), third (n = 5), fourth (n = 5), and eighth (n = 1) submissions. Acute kidney injury was reported in 32/32 dogs. Two of 32 dogs developed chronic kidney disease.

Conclusions and Clinical Importance: Persistent leptospiruria is common despite treatment with antibiotics frequently recommended for treatment. Follow-up urine PCR to confirm clearance of the organism is recommended in all dogs. In dogs with persistent leptospiruria, chronic kidney disease can develop after acute kidney injury.

KEYWORDS
antibiotic resistance, doxycycline, leptospirosis, leptospiruria

1 | INTRODUCTION

Leptospirosis, an important worldwide zoonotic disease, is recognized as a common cause of acute kidney injury in dogs and generally carries a favorable prognosis.[1-4] The diagnosis of leptospirosis in dogs is commonly obtained by the identification of pathogenic leptospires

Abbreviations: AKI, acute kidney injury; ALKP, serum alkaline phosphatase; KSVDL, Kansas State Veterinary Diagnostic Laboratory; MAT, microscopic agglutination test; PCR, polymerase chain reaction assay; PU/PD, polyuria/polydypsia.

in the urine or blood with the polymerase chain reaction assay (PCR) or by documenting a 4-fold rise in serum antibody titers over a 10- to 14-day period.[3,5] Point-of-care rapid IgG and IgM testing is available and is most commonly used to screen for disease with a confirmatory test recommended for definitive diagnosis.[6] Isolation of leptospires on culture of blood or urine is possible; however, culture is labor-intensive and isolation can take 8 to 12 weeks.[7] These factors make culture an infrequently utilized diagnostic.

Specific treatment for leptospirosis typically involves the use of amoxicillin or ampicillin early in the course of the disease followed by a course of doxycycline. Doxycycline is routinely used to eliminate organisms from the renal tubules and eliminate the potential for zoonotic transmission.[3,5] Additional PCR testing of the urine is not routinely performed during or after treatment to evaluate the efficacy of doxycycline in terminating leptospiruria on the assumption that treatment is routinely effective.[5] Long-term outcomes for dogs that survived leptospirosis are infrequently reported, but the findings of 2 studies suggested that persistent azotemia can be present in less than 6% or up to 18%.[8,9]

Persistent leptospiruria after antibiotic treatment occurs in people, dogs, and California sea lions.[10-13] Five dogs had persistent leptospiruria for 10 to 1059 days as determined by PCR results despite doxycycline (n = 4) or enrofloxacin (n = 2) treatment (1 dog had persistent leptospiruria after doxycycline and enrofloxacin treatment).[13] These dogs had a negative PCR on urine after the addition of enrofloxacin (n = 2) or clarithromycin (n = 3). Although long-term follow-up was not available for all 5 dogs in that study, all 5 had persistent azotemia (creatinine >1.4 mg/dL) when they were lost to follow-up (median 62 days; range, 10-1220 days). In contrast, California sea lions have persistent leptospiruria, as determined by PCR, even after the return of normal renal function.[12] Doxycycline was the only antibiotic associated with a negative urine PCR in California sea lions; however, only 4 of 6 that received doxycycline had a negative urine PCR at the time of release.

There are no published prospective studies that have evaluated the persistence of shedding of leptospires in the urine of dogs as evaluated by PCR in a clinical setting. Understanding the prevalence of persistent leptospuria, and potentially the presence of viable leptospires in the kidneys and urine during antibiotic treatment can have implications for altering treatment to improve survival and minimize morbidity. Additionally, there are no published guidelines to indicate the duration of use for personal protective equipment while treating dogs with leptospirosis. Understanding the persistence of shedding of leptospires can reduce the risk of zoonotic infection. We hypothesized that dogs would have a positive urine PCR if treated only with a penicillin derivative and that fewer than 10% of dogs would have a persistent urine PCR after receiving a minimum of 7 days of doxycycline. The purpose of this present study was to prospectively evaluate the duration of leptospiruria, as assessed by PCR, in client-owned dogs with leptospirosis and to determine which antibiotics were most commonly associated with termination of leptospiruria. When possible, outcomes were also assessed in relation to the duration of leptospiruria.

2 | MATERIALS AND METHODS

2.1 | Dogs

From April 2018 through May 2020, a prospective observational study of dogs with leptospirosis was performed. Dogs with leptospirosis were identified through the Kansas State Veterinary Diagnostic Laboratory (KSVDL; L. Peddireddi, J. Henningson) from submissions that returned a positive test result for the presence of pathogenic leptospires in urine as determined by PCR (n = 37 dogs). Submitting veterinarians and clients were offered additional urine PCR testing at no charge and case information was obtained with the signed consent of the dog owner (n = 33 dogs). Four dogs with a positive urine PCR did not submit any additional samples because of euthanasia (n = 3 dogs) or lack of interest in submitting additional samples (n = 1). One dog was lost to follow-up after the first study PCR was positive after 4 days of doxycycline, leaving 32 dogs with complete PCR data. Eight dogs were seen at the Kansas State University Veterinary Health Center (KSUVHC).

2.2 | Case information

Information recorded for each dog included age, sex, breed, city and state of residence, clinical presentation of leptospirosis (eg, acute kidney injury or chronic kidney disease, liver disease, polyuria/polydipsia [PU/PD], or pulmonary hemorrhagic syndrome), serum creatinine concentration, the results of the microscopic agglutination test (MAT) if performed, and the current antibiotic treatment (dose, route, and duration). Information recorded for each positive urine PCR result, and at the time the urine PCR became negative, included current antibiotic administration (and duration), serum creatinine concentration (if available), and outcome (eg, complete resolution, chronic kidney disease, or death).

2.3 | Follow-up testing and treatment protocol

After initial diagnosis, a fresh urine sample was collected from all dogs, as soon as possible, for repeat PCR testing at KSVDL. If the PCR was negative for pathogenic leptospires, participation in the study was complete. If the urine PCR was positive, the submitting veterinarian was contacted (K.R. Harkin) to ascertain the current clinical status of the dog and record the current antibiotic treatment. The next urine sample for PCR was collected 7 days after treatment to eliminate leptospiruria was initiated. If this urine PCR was negative, no additional PCR was performed. If the urine PCR was positive, the submitting veterinarian was contacted as previously noted. The submitting veterinarians were asked to submit fresh urine for PCR 7 days after each change in treatment or 7 days after the last positive PCR if no change in treatment was made. All treatment decisions were made by the submitting veterinarians who were given a list of suggested antibiotics (Table 1).

TABLE 1 Suggested antibiotics and dose to eliminate leptospiruria

Antibiotic	Dose recommended
Doxycycline	5 mg/kg PO q12h
Enrofloxacin	10 mg/kg PO q24h
Clarithromycin	10-12 mg/kg PO q12h
Cefdinir	10-15 mg/kg PO q12h
Doxycycline	10 mg/kg PO q12h

2.4 | Sample handling

Veterinarians were asked to collect a minimum of 5 mL of urine via cystocentesis or catheterization to avoid sample contamination (free-catch was discouraged but permitted if the dog was not amenable to the other methods of collection). The urine was refrigerated until it could be shipped overnight on ice to KSVDL. Samples collected Sunday through Thursday were shipped on ice within 24 hours of collection, but samples collected Friday or Saturday were refrigerated and shipped on ice Monday, barring holidays. Urine samples collected on dogs at the KSUVHC were submitted immediately to KSVDL for processing.

2.5 | Polymerase chain reaction assay

Leptospiral deoxyribonucleic acid extraction was performed in duplicate according to manufacturer directions using a commercial kit (QIAamp viral RNA kit, Qiagen Inc, Valencia, California). A 900 μL aliquot of urine was transferred to each of two 2.0 mL microcentrifuge tubes and centrifuged at 16 300*g* for 10 minutes. The supernatant was discarded and the pellet resuspended in 140 μL of 10 mM Tris-HCl pH 8 for use in the extraction kit. Polymerase chain reaction assay testing, which has been described in detail elsewhere, was performed on each sample with no modifications.[14]

3 | RESULTS

3.1 | Dogs

Thirty-two dogs were sampled until a negative urine PCR was obtained. The median age of dogs was 4 years (range, 2.5 months-14 years). There were 15 female (3 intact, 12 neutered) and 17 male (5 intact, 12 neutered) dogs. There were 16 breeds represented, including German shepherd dog (n = 4), boxer, Siberian husky, and miniature Schnauzer (n = 3, each), Shih tzu (n = 2) and 1 each of the following breeds: Basset hound, Belgian tervuren, Boston terrier, Cavalier King Charles spaniel, Havanese, Jack Russel terrier, Jindo, Maltese, Papillion, Pembroke Welsh corgi, and Yorkshire terrier. There were 6 dogs of mixed breeding. Patient samples were submitted from multiple states with 14 from Kansas, 8 from Nebraska, 4 from Missouri, 3 from Minnesota, 2 from Texas, and 1 from Virginia.

The attending veterinarian reported a clinical diagnosis of AKI in all 32 dogs. Serum creatinine concentration at the time of diagnosis was reported for 21 dogs (median 4.9 mg/dL; range, 1.7-8.4 mg/dL; reference interval, 0.5-1.4 mg/dL). Fourteen of 32 dogs (44%) were reported to have PU/PD and 2 dogs (6%) were reported to be oliguric (no dogs were anuric). Eight dogs (25%) were reported to have increased liver values and values were reported for 5 dogs. Serum alkaline phosphatase (ALKP) activity was increased in all 5 dogs (median 1333 U/L; range, 468-3085 U/L; reference interval, 10-130 U/L), bilirubin concentration was increased in 4 dogs (median 12.1 mg/dL; range, 3.4-24.0 mg/dL; reference interval, 0.0-0.2 mg/dL), and alanine transaminase activity was increased in 1 dog (421 U/L; reference interval, 20-144 U/L) that also had a concurrent increases in ALKP (468 U/L). Two dogs (6%) were reported to have anterior uveitis.

Microscopic agglutination test results were reported in 10 dogs. In 8 of 10 dogs, increased titers were reported on the first MAT. The highest reciprocal titer was to serovar Grippotyphosa in 5 dogs (12 800 in 3; 25 600 in 1; 51 200 in 1), Bratislava in 1 (1600), and Hardjo in 1 (400). One dog had a reciprocal titer of 12 800 to serovars Grippotyphosa, Pomona, and Bratislava. On the first MAT, 2 dogs had reciprocal titers of <100 to all 6 serovars. In 1 of these dogs, a convalescent titer was performed and showed a reciprocal titer of 3200 to serovars Grippotyphosa and Pomona. A point-of-care enzyme-linked immunosorbent assay (SNAP Lepto test, IDEXX, Westbrook, Maine) was reported positive on 2 dogs that did not have MAT performed.

3.2 | Days to first negative PCR after diagnosis

Fifteen of 32 dogs (47%) had a negative urine PCR on the first submission in the study. For these 15 dogs, urine samples were submitted 2 to 15 days (median 5 days) after the original PCR. Of these 15 dogs, 11 were treated with a single antibiotic and 4 were treated with a combination of antibiotics (Figure 1). In this group, an aminopenicillin was the only antibiotic administered to 5 of the dogs for a duration of 2 to 4 days (median 3 days) and a single dog was administered doxycycline for only 1 day in addition to being treated with an aminopenicillin for 6 days. Eight dogs received an antibiotic known to eliminate leptospiruria for a minimum of 3 days (doxycycline in 4 dogs for 4-9 days [median 6 days], ceftazidime in 2 dogs [3 and 8 days], and enrofloxacin in 2 dogs [3 and 5 days, both in combination with an aminopenicillin]). One dog received doxycycline alone for 1 day before the negative PCR.

Seventeen of 32 dogs (53%) had a positive PCR on the first submission in the study, with urine samples submitted 2 to 11 days (median 3 days) after the original PCR. At the time of the first PCR, 10 dogs had received doxycycline alone (n = 8 dogs) or in combination with an aminopenicillin (n = 2) for a range of 2 to 9 days (median 4). Other antibiotics administered include: enrofloxacin as a single agent for 3 days in 1 dog, enrofloxacin in combination with amoxicillin for 11 days in 1 dog, and ceftazidime as a single agent in 2 dogs for 2 and 3 days, respectively. Only 3 dogs had not received an antibiotic with the potential

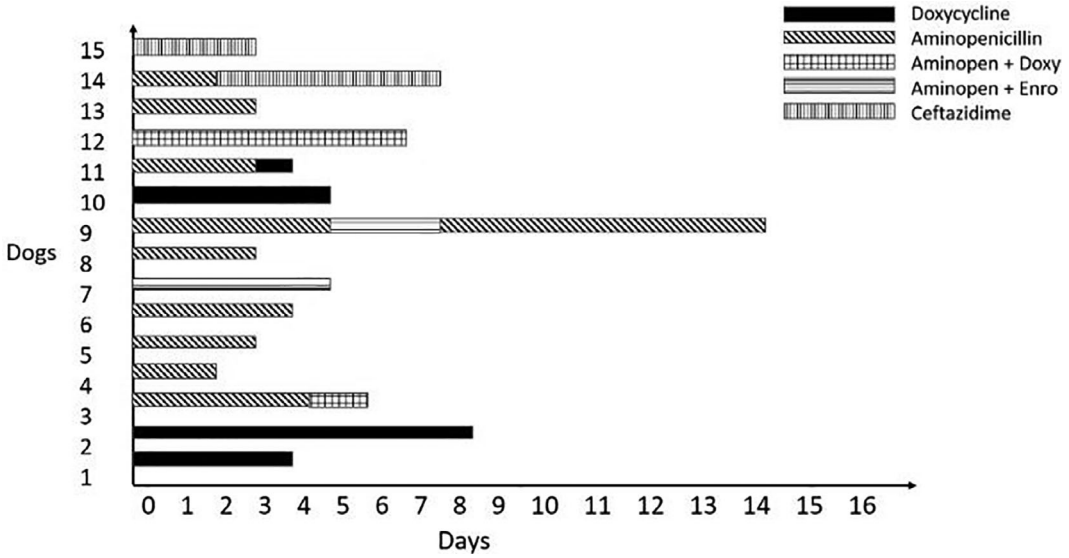

FIGURE 1 Dogs previously diagnosed with leptospirosis that tested negative at first follow-up PCR submission (n = 15). Median time to PCR submission was 5 days (range, 2-15 days). Antibiotic regimens are represented by patterns

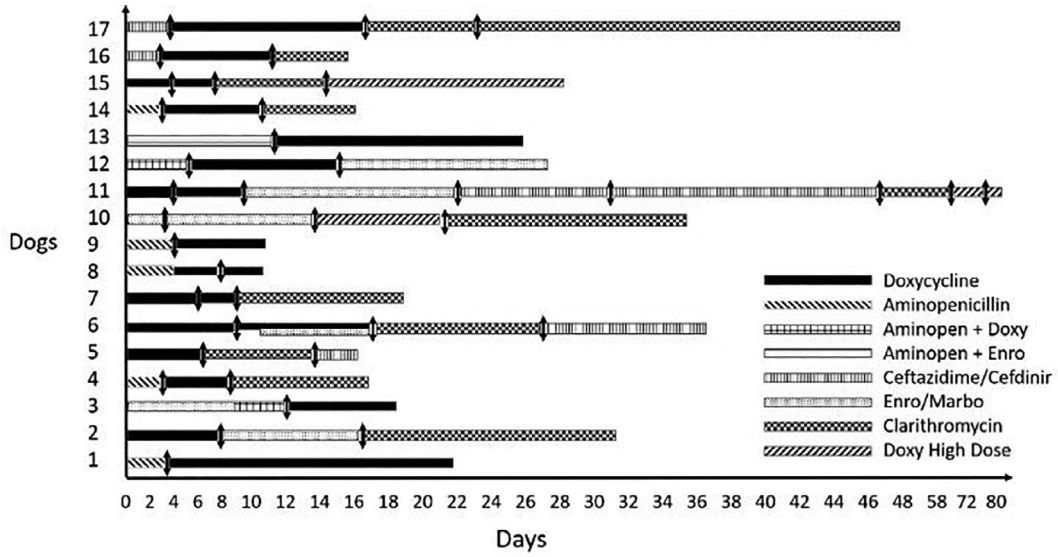

FIGURE 2 Time to negative PCR and antibiotic regime in dogs (n = 17) that did not clear leptospira with first treatment. Median time to negative PCR 22 days (range, 11-81 days). Antibiotic regimens are represented by patterns. Vertical double-headed arrow denotes positive urine PCR

to resolve leptospiruria at the time of the first PCR in the study (amoxicillin-clavulanic acid [n = 2 dogs] and ampicillin [n = 1]). A summary of the antibiotic treatment for these 17 dogs is shown in Figure 2.

The urine PCR was negative on the second submission in 6 dogs with samples submitted in 3 to 17 days (median 7 days) (Figure 2). Five dogs received doxycycline that lasted 6 to 17 days (median 10 days). The sixth dog received clarithromycin for 8 days after having a positive PCR after 7 days of treatment with doxycycline.

The urine PCR was negative on the third submission in 5 dogs with samples submitted in 7 to 14 days (median 11 days) (Figure 2.). Four of these dogs had a negative urine PCR after receiving clarithromycin and 1 dog after receiving enrofloxacin. All 5 of these dogs failed to achieve a negative urine PCR with doxycycline.

The urine PCR was negative on the fourth submission in 5 dogs with samples submitted in 3 to 25 days (median 11 days) (Figure 2). These dogs were successfully treated with clarithromycin (n = 2 dogs), doxycycline at 7.5 mg/kg (n = 1), cefdinir (n = 1), and ceftazidime (n = 1). Doxycycline failed to result in a negative PCR in all 5 dogs at a standard dose. Clarithromycin also failed to produce a negative PCR in the 2 dogs that were successfully treated with 3rd generation cephalosporins. The last dog achieved a negative PCR on the 8th submission after 21 days of doxycycline at 10 mg/kg PO

q12h after unsuccessful treatment with doxycycline at 5 mg/kg, enrofloxacin, cefdinir, and clarithromycin (Figure 2).

3.3 | Antibiotic regimens

Doxycycline at 5 mg/kg PO q12h in 11 dogs and at 10 mg/kg PO q12h in 1 dog was not associated with a negative PCR. However, in 2 dogs a higher dose of doxycycline (7.5 and 10 mg/kg PO q12h, respectively) was temporally associated with a negative PCR after 9 and 21 days, respectively. The dog that required 21 days of doxycycline at 10 mg/kg PO q12h had a positive PCR after 14 days, but negative 7 days later. Doxycycline was continued in that dog as the cycle threshold of the PCR had increased for the first time after 14 days of treatment (Ct of 9-10 for all previous PCR and 14 at the last positive). In 1 dog, the PCR remained positive after 6 days of doxycycline treatment, but doxycycline was discontinued due to worsening azotemia. This dog also had a positive PCR and worsening disease after 7 days of clarithromycin and only improved after treatment with ceftazidime (negative PCR after 3 days). In another dog, doxycycline was discontinued after 8 days due to suspected hepatotoxicity from doxycycline (urine PCR was positive at this time). The PCR was negative and the suspected hepatotoxicity resolved in this dog after 7 days of clarithromycin. Doxycycline was not associated with a negative PCR in the 2 dogs that had a positive PCR after initial ceftazidime treatment.

Clarithromycin was temporally associated with a negative PCR over 7 to 32 days (median 11 days) in 7 of these 17 dogs after they failed doxycycline (n = 3 dogs), ceftazidime followed by doxycycline (n = 1), doxycycline followed by marbofloxacin (n = 1), enrofloxacin followed by doxycycline (n = 1), and doxycycline preceded by ceftazidime (n = 1). Clarithromycin was not associated with a negative PCR in 4 dogs after 7 to 12 days (median 10 days). These 4 dogs all failed doxycycline initially, but 2 of them subsequently had a negative PCR on a higher dose of doxycycline (see above). The other 2 dogs subsequently developed a negative PCR after either ceftazidime (3 days) or cefdinir (10 days). Enrofloxacin was temporally associated with a negative PCR in 1 dog (12 days) after failed doxycycline treatment (13 days). Fluoroquinolone treatment was not associated with a negative PCR in 5 dogs treated with enrofloxacin and 1 dog treated with marbofloxacin.

3.4 | Clinical outcomes

Short-term survival (up to 30 days) was known for all dogs with a case fatality rate of 3% (1 of 32 dogs). Specific outcomes (eg, progression or resolution of azotemia) were available for 23 dogs. Ten of these dogs were negative on the first PCR in the study and 9 dogs had rapid resolution of azotemia. One dog, a German shepherd dog, was negative on the first PCR in the study after 3 days of ceftazidime, was oliguric for the first 72 hours of hospitalization (highest creatinine of 13.1 mg/dL on day 4) and was euthanized after 10 days of hospitalization with a creatinine of 7.8 mg/dL. Follow-up was available on 13 dogs that were still positive at the first PCR. Five dogs were reported to have achieved a normal creatinine from 7 to 79 days (median 21 days), although only 1 had resolved azotemia in less than 17 days and this dog achieved a negative PCR after 7 days of doxycycline, having received only amoxicillin-clavulanic acid for 5 days before to the first study PCR. Six dogs still had an increased creatinine (median 2.2 mg/dL; range, 1.7-3.1 mg/dL) at the last reported measurement (median 15 days; range, 5-29 days) before they were lost to follow-up. Two dogs had persistent azotemia at 103 and 181 days after the diagnosis of leptospirosis, with a final reported creatinine of 1.7 and 2.2, respectively. One of these 2 dogs (creatinine 2.2 mg/dL at 181 days) was a spayed female German shepherd dog that remained PCR positive while administered doxycycline and clarithromycin and had extreme polyuria and worsening azotemia with IV fluid therapy until ceftazidime was administered. This dog had uveitis on admission, but also developed hypertension and retinal detachment while administered doxycycline.

Breed and city of residence were compared to the persistence of a positive PCR. Breeds that were represented by more than 1 dog were distributed in both groups, except for 2 breeds: miniature Schnauzer and German shepherd dog. All 3 miniature Schnauzers achieved a negative PCR within 2 to 13 days of doxycycline and had resolution of azotemia, even in 1 dog that was clinical for 8 days before initiating doxycycline treatment. All 3 German shepherd dogs that survived had persistently positive PCRs after doxycycline at the standard dose and 2 were known to have residual azotemia after achieving a negative PCR (the long-term outcome of the third was unknown). The 1 German shepherd dog that became PCR negative promptly with ceftazidime did not survive. Likewise, cities that were represented by more than 1 dog were distributed in both groups, except for 4 cities. All dogs from Lincoln, Nebraska (n = 3 dogs) and Leawood, Kansas (n = 2) failed to achieve a negative PCR on doxycycline. All dogs from the Minneapolis, Minnesota metropolitan area (n = 3 dogs) and Kansas City, Missouri (n = 4) rapidly achieved a negative PCR and had rapid resolution of azotemia, including 5 that resolved with an aminopenicillin alone. This study was not large enough to reach statistically significant conclusions regarding city/state and breed.

4 | DISCUSSION

The results of this study show that although 20 of 32 dogs (63%) achieved a negative urine PCR, in 14 days or less, with antibiotics that are expected to resolve leptospiruria, 12 dogs (38%) still had a positive urine PCR after doxycycline treatment with treatment that lasted as long as 18 days. These results raise similar concerns as previous reports regarding persistent leptospiruria in dogs despite doxycycline treatment.[11,13] While it is possible that prolonged doxycycline treatment time could have improved the response to doxycycline, only 1 dog received fewer than 7 days of doxycycline and 6 received 9 days or more. In comparison, 5 of the 9 dogs with a negative PCR

after doxycycline as first-line treatment received it for 7 days or less and 2 received it for only 4 and 5 days, respectively.

For the dogs reported here, the recommendation for repeat urine PCR testing after 7 days was predicated on the belief that leptospires should be cleared from the kidneys after 7 days of appropriate antibiotic treatment, resulting in a negative urine PCR. To the authors' knowledge, there are no published studies that have assessed time to clearance of leptospires from target organs in dogs. However, using a Syrian hamster model, a negative PCR occurred after treatment with doxycycline (10 mg/kg once) within 2 to 3 days.[15] Similarly, a single injection of streptomycin or dihydrostreptomycin given to cattle experimentally or naturally infected with leptospirosis resulted in a negative urine PCR within 7 days.[16,17] Last, a study utilizing a hamster model, which evaluated the efficacy of cefepime, ertapenem, and norfloxacin, PCR results had a high correlation with culture results.[18] These results suggest that nonviable leptospires are cleared rapidly from target organs and that residual, nonviable genetic material is an unlikely cause of persistent PCR positive results. While effete organism could theoretically play a role in positive PCR results, it was the expectation of the authors that leptospires would be eliminated from the kidneys in dogs in a similar fashion as described in the studies above.

A number of studies comparing antibiotic efficacy in people have shown similar equivalent success with penicillins, doxycycline, azithromycin, and cephalosporins with mortality or resolution of fever being the primary outcomes assessed.[19-21] If acute morbidity was the primary outcome for dogs with an intent-to-treat in the current study, all antibiotics used would have demonstrated efficacy. With the primary outcome defined as obtaining a negative urine PCR after antibiotic treatment, the current study demonstrated variable success and failure of every antibiotic used in these dogs.

Doxycycline and clarithromycin appeared to be the most reliable oral antibiotics to be associated with a negative urine PCR; however, neither was routinely successful. Surprisingly, 2 dogs that failed to achieve a negative PCR with doxycycline at the standard dose of 5 mg/kg PO q12h did so at a higher dose. The reason for this response at the higher dose is unclear; however, poor oral absorption seems unlikely. Doxycycline reaches concentrations in the urine at least 10-fold higher than serum in dogs and would be expected to effectively clear leptospiruria if isolates are susceptible.[22] While doxycycline concentrates in the urine of healthy dogs, dogs with decreased glomerular filtration rate might not have the same ability. While not established in dogs, humans with reduced renal glomerular filtration rate can have decreased excretion of doxycycline.[23] In this group of dogs with AKI, higher doses of doxycycline could increase the urinary concentration resulting in a response to treatment. The authors are unaware of a similar study on clarithromycin in dogs. However, a study in foals showed that clarithromycin reached concentrations in urine in excess of 10-fold that of serum.[24]

In our dogs that were unable to be administered doxycycline PO, ceftazidime, which was administered IV, was associated with a negative PCR in 3 dogs, but failed in 2 other dogs. However, the treatment duration was brief in those dogs (2 and 3 days) as they were transitioned to oral doxycycline once their condition improved. Whether additional days of ceftazidime treatment could have correlated with a negative PCR in those 2 dogs is uncertain. Fluoroquinolones were routinely unsuccessful in this group of dogs, in contrast to the results of a recent study.[25] It is possible that geographical variables in the strains of *Leptospira* spp. contribute to this finding.

An unexpected finding in this study was that 7 dogs had a negative urine PCR having received an aminopenicillin alone (n = 5 dogs) or in combination with fewer than 3 days of doxycycline (n = 2). Penicillin and aminopenicillins are recommended for first-line treatment in dogs with leptospirosis for the initial leptospiremic phase, but it is widely believed that they do not clear renal tubules of leptospires.[3,5,15] After a transient intracellular phase, leptospires attach to the basement membrane in the renal tubular space where aminopenicillins reach therapeutic levels.[26,27] Therefore, aminopenicillins should theoretically resolve leptospiruria. Previous in vitro studies have reported only a small number of leptospira isolates with minimum inhibitory concentration values in a range that could suggest antibiotic resistance in clinical animals.[28-30] Failure of aminopenicillins and other antibiotics to routinely eliminate leptospiruria, despite their apparent efficacy in eliminating leptospiremia, could suggest that factors other than urinary concentrations of antibiotics impact resolution of leptospiruria.

A study using live-imaging of bioluminescent leptospires in mice demonstrated that infective doses of 10^6 to 10^7 bacteria were cleared without antimicrobial treatment and that a higher infective dose (2×10^8) was fatal within 3 days.[31] Although infective dose likely would not impact the response to antibiotic susceptibility, a delay in antibiotic administration and creation of an established renal tubular infection could adversely affect response to antibiotics. In the same study, treatment with penicillin G, ciprofloxacin, or azithromycin given from 1 to 5 days after inoculation effectively cleared leptospiral infection, but a delay in administration resulted in a chronic renal tubular infection that could be reduced but not cleared with the same antibiotics.[31] The majority of the dogs in the current study were administered appropriate antibiotics while PCR was pending to confirm a diagnosis. However, dogs were sick for a variable period of time prior to presentation to their veterinarian and this could have impacted the response to antibiotics.

It is also possible that factors beyond physical localization in the renal tubules alone could be responsible for apparent antibiotic resistance in established infections. Although many common pathogenic *Leptospira* serovars carry the genes that code for multiple-drug efflux transporters and antimicrobial/multidrug resistance, the mechanisms that drive their expression are unknown.[32,33] One study demonstrated that temperature and osmolarity affected gene and antigen expression *in vitro* in pathogenic *Leptospira* and a second study demonstrated differential antigen expression in serovar Copenhageni isolates cultured *in vitro* to those isolated from the urine of experimentally infected rats.[34-36] Whether the microenvironment or the process of renal colonization stimulates the expression of genes resulting in antibiotic resistance in pathogenic *Leptospira* spp. is unknown.

Limitations to this study include the lack of historical clinical data in some dogs. While this limited our ability to determine the baseline

serum creatinine in all dogs, AKI was reported by the submitting veterinarians as the manifestation of leptospirosis in all of these dogs and the median creatinine was 4.9 g/dL in the 21 dogs with a recorded value. As these cases were managed by the submitting veterinarian, repeat PCR testing was performed at slightly different time points based on owner and veterinarian schedules. This dynamic also affected the choice of antibiotic protocol and the amount of follow-up ancillary testing (convalescent titers, recheck chemistry panels) that was performed. Despite these confounding factors, it is still clear that many dogs did not achieve a negative urine PCR in the face of standard of care for treatment of leptospirosis.

Failure of the antibiotics used in the dogs in this study to routinely correlate with a negative urine PCR increases the concern for antibiotic resistance in leptospirosis, which has implications for treatment, morbidity, and case fatality. Failure to clear the organism in a timely manner can lead to development of chronic kidney disease as was seen in 2 of the dogs in this study with persistently increased serum creatinine concentrations. The development of chronic kidney disease after leptospirosis infection has been previously documented in people and dogs.[37,38]Additionally, there is likely a subset of dogs who develop chronic kidney disease, which is not recognized because renal function is still above the threshold for development of increased serum creatinine concentrations. Given the zoonotic nature of *Leptospira* spp., persistent leptospiruria can also endanger those in contact with infected dogs. Possible future studies including a more regimented treatment and testing protocol which could offer more support for specific antibiotic treatment in dogs that fail to resolve leptospiruria with standard doxycycline treatment and to document the viability of leptospires in these dogs with persistently positive PCRs.

In conclusion, the results of this study support the need for follow-up urine PCR to confirm clearance of organisms from the urine in dogs with leptospirosis. Failure to clear the organism in a timely manner should be addressed with a change in antibiotic treatment as persistent leptospiruria can lead to development of chronic kidney disease and an increased risk of zoonotic infection. Although the mechanism behind persistent leptospiruria despite antibiotic treatment is not known, the possibility for antibiotic resistance in *Leptospira* spp. should be considered.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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.

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SUPPORTING INFORMATION

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