Clinical application of a polymerase chain reaction assay for diagnosis of leptospirosis in dogs

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Objective—To evaluate the use of a polymerase chain reaction (PCR) assay on urine samples for diagnosis of leptospirosis in dogs.

Design—Prospective case study.

Animals—132 dogs with clinical signs suggestive of leptospirosis and 13 healthy dogs.

Procedure—PCR testing was performed on urine samples to detect leptospiral DNA; results were compared with results of conventional criteria for the diagnosis of leptospirosis.

Results—Leptospirosis was diagnosed in 8 dogs via established criteria; all these dogs had positive results of PCR assay, including 1 dog with positive results before seroconversion developed. A positive PCR assay result was also obtained in 16 dogs that did not have a confirmed diagnosis of leptospirosis. In the 8 dogs that had a confirmed diagnosis of leptospirosis, serovars pomona (n = 3 dogs), grippotyphosa (2), canicola (2), and bratislava (1) were identified serologically. The remaining 121 dogs all had a diagnosis other than leptospirosis or were healthy. For PCR testing on urine, sensitivity was 100%, specificity was 88.9%, positive predictive value was 55%, and negative predictive value was 100%.

Conclusions and Clinical Relevance—Positive PCR test results prior to seroconversion may have value in establishing an early diagnosis. Positive results in dogs that had signs consistent with leptospirosis despite failing to meet established criteria for leptospirosis raise questions regarding the sensitivity of serologic testing in diagnosis of leptospirosis. Serovars pomona, grippotyphosa, and canicola were most common. (J Am Vet Med Assoc 2003;222:1224–1229)

Recent concerns regarding leptospirosis in dogs have focused on the possibility of increasing prevalence of the disease and a shift in serovars responsible for disease. Results of several studies indicate that serovars pomona, grippotyphosa, and bratislava are presently prevalent and responsible for most cases of canine leptospirosis. Results of 1 study suggest that serovars grippotyphosa and pomona have been predominant in 1 region for at least 16 years and that there likely has not been a major change. The number of articles published during the last 6 years documenting clinical cases of leptospirosis may lead to suspicion that the disease is a reemerging zoonosis; however, this may also reflect a greater awareness of the disease and more rigorous attempts to diagnose it.

The common clinical signs expected with leptospiral infections in dogs include signs of depression, anorexia, vomiting, diarrhea, ocular discharge, signs of renal pain, myalgia, arthralgia, and icterus. These clinical signs are typically associated with acute renal failure, acute cholestatic liver disease, and hemolysis. Uncommon clinical signs include pulmonary hemorrhage, nonanatomic polyuria and polydipsia, and pleural effusion. Uncommon signs of human leptospirosis include acute pancreatitis, pulmonary hemorrhage, and aseptic meningitis. The diagnosis may be overlooked in such atypical cases and is also frequently missed in more classic cases if the clinician does not maintain an index of suspicion for leptospirosis.

Diagnosis of leptospirosis is typically accomplished ante mortem through identification of a 4-fold increase in antibody titers with the microscopic agglutination test or by finding a single high titer with compatible clinical signs. Most laboratories in the United States test for 6 serovars (canicola, icterohemorrhagiae, grippotyphosa, pomona, hardjo, and bratislava), although a few omit serovar hardjo, and serovar autumnalis is included in others. Depending on the size of the inoculum, titers may develop as early as day 5 after inoculation or may be delayed 13 days or longer. Two studies detected seronegative dogs during the acute phase of infection, with conversion at the 2-week convalescent period, suggesting that certain dogs may not receive a correct diagnosis if convalescent titers are not evaluated. In addition, dogs may be actively infected and shedding organisms, serving as a zoonotic risk, yet be seronegative and clinically normal.

Additional methods used in the diagnosis of leptospirosis include darkfield microscopy, bacteriologic culture, and fluorescent antibody testing. Darkfield microscopy has poor sensitivity and is rarely used as a routine diagnostic test. Bacteriologic culture of urine or blood for leptospires is also a low-yield procedure due to the difficulty in culturing the organism and the lengthy duration of culture (12 to 16 weeks). Fluorescent antibody (FA) testing can be performed on urine, blood, and histologic tissue specimens. The FA test has good sensitivity, although in 1 study, 4 of 4 dogs experimentally infected with leptospires had negative results of FA tests on urine, and only 1 of 4 dogs with naturally occurring disease had a positive FA test result with urine. In addition, only 2 of 6 dogs with naturally occurring disease had a positive FA test result with renal or liver tissue.

Application of the polymerase chain reaction
(PCR) assay for the diagnosis of leptospirosis in horses, cattle, dogs, and humans has been described. Clinical samples tested included urine, blood, aqueous humor, semen, and CSF. The PCR assay has the advantage of identifying an infection earlier than serologic testing, although the sensitivity of the assay may result in false-positive diagnoses.

The purpose of the study reported here was to evaluate the use of PCR testing on urine as a diagnostic tool for leptospirosis in dogs.

**Materials and Methods**

**Case selection**—One hundred forty-five dogs evaluated during a 24-month period by the internal medicine, community practice, emergency, or oncology services at Kansas State University Veterinary Medical Teaching Hospital (KSU-VMTH) were evaluated. Dogs (n = 132) were chosen predominantly if they had clinical signs or laboratory findings that have been described for canine leptospirosis. Also included were 13 apparently healthy dogs or dogs with unrelated diseases. This group consisted of 10 dogs owned by the staff at the KSU-VMTH; 1 seronegative dog that had familial epilepsy and was tested during boarding at KSU-VMTH, yielding positive results of the PCR assay; the healthy housemate of the dog with epilepsy; and a dog that had severe otitis externa and was tested solely because it was a German Shepherd Dog, a breed that may have higher risk for leptospirosis than other breeds. All procedures were approved by either the Institutional Biosafety Committee or Institutional Animal Care and Use Committee at Kansas State University.

**Serologic examination**—The microscopic agglutination test was performed for serovars canicola, bratislava, pomona,icterohemorrhagiae, hardjo, and grippotyphosa. Results were recorded as seronegative if the titer was < 1:100. For reporting, the serovar with the highest titer was recorded as the important serovar and was the only serovar reported for that dog unless multiple serovars had equivalent high tiers.

**Sample collection and DNA isolation**—Urine samples (6 to 20 mL) were collected by first watch, cystocentesis, or catheterization and stored in a sterile plastic urine cup or sterile red-topped blood collection tube. Urine was refrigerated for a maximum of 48 hours prior to processing. Urine was transferred to a sterile 30-mL plastic centrifuge tube and centrifuged at 15,000 rpm for 20 minutes. The supernatant was transferred to a second sterile 30-mL centrifuge tube and centrifuged at 15,000 rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL of phosphate-buffered saline solution. From this suspension, 60 µL was used for DNA isolation, and the remainder was stored at –80°C. The DNA isolation was performed by use of a kit according to the manufacturer's instructions.

**Primer selection**—Genus-specific primers for the PCR assay were selected from published primers that amplified a 482-bp fragment of the 23S rDNA. Primers were prepared by a commercial company and designated L737 and L1218 for the upstream and downstream primers, respectively.

**PCR assay**—The PCR assay was performed with 15 µL of purified DNA, 25 µL of Taq polymerase, 5 µL of primer L737, and 5 µL of primer L1281. All reactions were performed with a thermocycler under the following conditions: 95°C for 10 minutes for melting, 35 cycles at 96°C for 1 minute for denaturing, 60°C for 1 minute for annealing, 72°C for 1 minute for extension, and 72°C for 10 minutes for the final extension sequence. A negative and positive control test were performed with each run or per 6 clinical samples, whichever was less. A negative control test was performed with 15 µL of sterile water in place of purified DNA, and a positive control test was performed with 15 µL of purified DNA from a stock culture of *Leptospira kirschneri* serovar grippotyphosa. After amplification, 20 µL of each sample, including the control samples, was evaluated by use of electrophoresis on a 1.5% gel, with a 100-bp ladder run concurrently. Samples with positive results were further evaluated for specificity.

**Determination of specificity**—Confirmation of the identification of the 482-bp band was performed with restriction endonuclease *Apa I*, which recognizes the sequence 5'-GGGCCC-3' and was predicted to produce fragments of approximately 260 and 220 bp only in pathogenic leptospires. After digestion, 15 µL of each sample was evaluated by use of electrophoresis on a 1.5% gel, with a 100-bp ladder run concurrently. The PCR and restriction endonuclease activity were evaluated on pathogenic serovars of leptospires including canicola, icterohemorrhagiae, bratislava, grippotyphosa, pomona, hardjo, bataliae, australis, autumnalis, hebdomadis, ballum, sejroe, pyrogenes, tarassovi, and zwajczak, and nonpathogenic serovars including patoc and billyeck.

**Statistical analyses**—Sensitivity, specificity, negative predictive value, and positive predictive value were determined on the basis of criteria for a diagnosis of leptospirosis, including a single titer of ≥ 1:400 with appropriate clinical signs or a 4-fold increase in the convalescent titer at 2 or 4 weeks.

**Results**

The PCR reaction amplified DNA from all leptospirosera tested, including the 2 nonpathogenic serovars. After samples were digested by use of restriction endonuclease, all pathogenic serovars yielded the predicted 260- and 220-bp fragments, whereas the nonpathogenic serovars did not.

The numbers of true and false results, based on our criteria for a diagnosis of leptospirosis, were determined (Table 1). Of the 145 dogs, 121 had negative PCR test results on urine and did not meet criteria for a diagnosis of leptospirosis; 11 dogs had a definitive diagnosis other than leptospirosis, whereas 10 dogs were healthy and owned by members of the KSU-VMTH clinical staff. A 482-bp fragment was not identified via gel electrophoresis from any of these samples, so additional *Apa I* digestions were not necessary. The diseases were classified in organ system groupings, which included urinary tract disease (n = 36), gastrointestinal tract disease (15), hepatic disease (9), endocrine disease (9), neurologic disease (8), immune-mediated disease (7), neoplastic disease (14), cardiac disease (2), and miscellaneous diseases (11).

Of the 36 dogs with negative PCR assay results that had urinary tract diseases, 10 dogs had lower urinary tract infections either with *Streptococcus* spp (n = 4 dogs), *Escherichia coli* (3), *Klebsiella* spp (2), or...
Proteus spp (1). Chronic renal failure was diagnosed in 12 dogs, and acute renal failure was diagnosed in 5 dogs. An underlying etiology for the chronic renal failure in 9 dogs was not determined, although serologic testing yielded negative results in these dogs. Two dogs with chronic renal failure had amyloidosis, and 1 dog had renal dysplasia. Acute renal failure was a result of pyelonephritis (n = 2 dogs) or toxicoses (3). Undiagnosed polyuria (n = 3 dogs), urinary incontinence secondary to sphincter incompetence (3), renal insufficiency (2), and protein-losing glomerulopathy (1) were seen in the remaining dogs with urinary tract disease. In all other categories, the next most commonly diagnosed diseases were acute pancreatitis (n = 4 dogs) and vomiting secondary to dietary indiscretion (4).

Samples from 24 dogs yielded a positive PCR test result, 14 of which were false positives, as judged by our conventional criteria for diagnosis. Acute renal failure was identified in 11 dogs, and positive titers were obtained in 5 of these dogs. Three of these dogs had positive titers to serovar pomona (1:400, 1:800, and 1:800), although 1 of these dogs was seronegative at initial evaluation when PCR assay results were positive and had a positive titer (1:800) at the 2-week convalescent evaluation. Convalescent titers were not obtained in the dog with a titer of 1:400, and the remaining dog with acute titers of 1:800 died 1 week later with signs compatible with meningoencephalitis; a necropsy was not performed. One dog had a titer to serovar canicola (1:400) at the acute stage, was seronegative to serovar icterohemorrhagiae, and had no history of vaccination for leptospirosis. This dog died 1 week later with signs of meningoencephalitis. At necropsy, mesangiotropic proliferative glomerulonephritis was identified on histologic evaluation of the kidneys. No lesions were detected in the brain. The last of these 5 dogs with positive titers had an acute titer of 1:12,800 to serovar bratislava.

The diagnosis of leptospirosis was suspected, but not confirmed, in 2 dogs with positive results of PCR, both of which were seronegative because of neutrophic tubulointerstitial nephritis on histologic examination. However, leptospires were not identified by silver staining or fluorescent antibody testing on kidney tissue.

One dog was seronegative on multiple occasions during a period of several months but had complete resolution of mild acute renal failure with doxycycline administration. On the basis of results of all other diagnostic testing, including bacteriologic cultures of urine, no other diagnosis had been suggested in this dog. One dog was seronegative and the owner did not return for follow-up, although acute renal failure resolved with IV administration of fluids and administration of ampicillin and doxycycline. The owners of 2 dogs declined all additional diagnostic tests, the dogs were euthanatized, and necropsy was not performed. One of these dogs also had acute cholestatic hepatic disease, consistent with leptospirosis.

Three dogs with positive results of PCR had chronic renal failure; titers were positive at low concentrations to serovar hardjo in 2 dogs (1:100 and 1:200), and the third was seronegative. Histologic evaluation of renal biopsy specimens obtained with ultrasonographic guidance in the dog with the 1:100 titer revealed normal findings on 2 occasions. The dog with the 1:200 titer died at home 2 weeks after surgery for pyometra and removal of a pin that was embedded in the pancreas and cecum. A necropsy was not performed and renal biopsy specimens were not obtained at surgery. The seronegative dog was euthanatized 3 months after initial diagnosis. Histologic evaluation of samples obtained at necropsy identified membranoproliferative glomerulopathy with interstitial nephritis and suppurative cholangiohepatitis. This dog lived with a prairie dog that had been domesticated but died a few months before the initial diagnosis of chronic renal failure in the dog.

Three dogs with positive results of PCR were polyuric and polydipsic, although extensive diagnostic testing had not established a diagnosis prior to evaluation at the KSU-VMT. None of these dogs were azotemic, and all of them had rapid resolution of polyuria and polydipsia receiving doxycycline. One dog had a titer of 1:3,200 to serovar grippotyphosa, 1 had a titer of 1:1,600 to canicola with no history of vaccination, and the third dog was seronegative via acute and convalescent titer examinations.

A 7-year-old male Rat Terrier evaluated for recurrent high fever (40 to 41°C [104 to 106°F]) during a 3-day period had positive results of PCR testing. Results of CBC, serum biochemical profile, urinalysis, and bacteriologic culture of urine were all within reference ranges, and serologic testing revealed a titer of 1:12,800 to serovars grippotyphosa and bratislava. The convalescent titer to serovar grippotyphosa remained high at 1:12,800, whereas the titer to serovar bratislava decreased to 1:6,400. This dog responded quickly and completely to doxycycline administration.

A positive PCR test result was obtained in a 5-year-old male Newfoundland evaluated for a history of progressive hind limb paresis during a 2- to 4-week period. This dog was nonambulatory at evaluation, with upper motor neuron signs to the hind limbs. The dog had mild thrombocytopenia (134,000 cells/µL; reference range, 150,000 to 400,000 cells/µL) and was seronegative to *Ehrlichia canis* and *Rickettsia rickettsii*. Uveitis was diagnosed by the ophthalmology service, and a CSF sample obtained via lumbar puncture had high protein concentration (161 g/dL; reference range, <45 g/dL), WBC concentration of 8 cells/µL (all lymphocytes; reference range, <5 cells/µL), negative results of bacteriologic culture, and negative results for antibodies against canine distemper virus. This dog improved rapidly with doxycycline administration and was ambulatory within a week of evaluation. The dog was seronegative for leptospirosis at the time of evaluation, and convalescent samples were never obtained.

The PCR test results were positive in 2 dogs that were apparently healthy housemates. One dog had familial epilepsy and was tested as a screening procedure during boarding. This dog had a positive PCR test result on 2 occasions 2 weeks apart and negative PCR test results 2 weeks after doxycycline administration. The other dog also was treated with doxycycline and had negative PCR test results 2 weeks later. Both dogs
were active in rodent control on the farm at which they resided, and both were seronegative when tested at initial evaluation.

A German Shepherd Dog had no clinical evidence of leptospirosis, being evaluated for bilateral total ear canal ablations, and had a positive PCR test result. This dog was never treated for leptospiral infection, was seronegative, and no follow-up samples for PCR testing were obtained. Two dogs being treated or evaluated for lymphosarcoma, an Irish Setter and a German Shepherd Dog, also tested positive by use of the PCR assay. The German Shepherd Dog tested positive at the time of diagnosis of lymphosarcoma, but the owner chose euthanasia and declined a necropsy. The Irish Setter had positive results of PCR assay 3 months into the chemotherapeutic protocol when it was evaluated for acute hepatitis. The liver disease resolved with supportive care, including ampicillin and doxycycline administration; however, serum antibodies were never detected.

A diagnosis of leptospirosis was made in 8 dogs on the basis of the established criteria. The serovars with the highest titers in these dogs were pomona (n = 3 dogs), grippotyphosa (2), canicola (2), and bratislava (1). No dogs had a diagnosis of leptospirosis without a positive PCR test result. There were 121 dogs with negative PCR test results that did not have leptospirosis and 16 dogs that had a positive PCR test result but did not meet the criteria for having leptospirosis. With our criteria, the PCR test had a sensitivity of 100% and a specificity of 88.3%. The positive predictive value of the test was 33%, and the negative predictive value of the test was 100%. Prevalence of leptospirosis in dogs with appropriate clinical signs or laboratory findings was calculated to be 5.5% during the 24-month period.

**Discussion**

In this study, every attempt was made to evaluate all dogs with clinical signs or laboratory findings that have been described with leptospirosis. However, it is likely that many dogs with compatible clinical signs received a diagnosis other than leptospirosis within the first day of hospitalization by a clinician unaware of the study and were treated and released without the investigators in this study being notified. Determination of prevalence in this study was likely affected by the omission of these cases. In addition, there were several dogs included in the study that did not have clinical signs compatible with leptospirosis. Among these were 10 clinically normal dogs owned by staff at the KSU-VMTH, 2 other apparently healthy dogs known to be active in rodent control, and 1 German Shepherd Dog that was evaluated because of an apparently high risk in this breed.

The primers for the 23S rRNA were chosen because they were developed from 33 serovars of *Leptospira* spp and had the best chance of identifying all serovars that might be encountered. The primer pair and restriction endonuclease were tested against 15 pathogenic and 2 nonpathogenic serovars and yielded the anticipated results, suggesting that the testing method was appropriate. Boiling is frequently used as a method of DNA extraction for leptospires, although this method was not selected for our study, because results of a previous study suggest that boiling may affect the results of the PCR assay. Urine samples were allowed to remain in a refrigerator for up to 48 hours prior to processing, which could theoretically affect DNA recovery.

Clinically ill dogs with negative PCR assay results had a wide spectrum of diseases. All of these dogs had clinical signs or laboratory findings that were consistent with leptospirosis. Testing dogs with clinical signs of leptospirosis was important in evaluation of the test to determine the possibility of false-positive results.

The 8 dogs with confirmed leptospirosis included 5 dogs with acute renal failure, 2 dogs with polyuria and polydipsia, and 1 dog with fever. The serologic findings in these 8 dogs revealed that serovar pomona was most common, although serovars grippotyphosa and canicola were also common. Serovar bratislava was the only other serovar detected. In 1 dog, the PCR test result was positive before serologic conversion, indicating the potential usefulness of PCR testing in the early stages of infection. Although finding serovars pomona, grippotyphosa, and bratislava was not unexpected, finding serovar canicola infections in 2 nonvaccinated dogs was a surprise. This suggests that serovar canicola is still a potential cause of leptospirosis in dogs, and vaccination of at-risk dogs should not be discontinued because of a perceived risk of vaccine reactions.

The gold standard for diagnosis in these dogs was fairly liberal, with a titer of ≥1:400 and compatible clinical signs as 2 of the accepted criteria for diagnosis. Despite that, however, 16 dogs had positive results of PCR testing yet failed to meet the criteria for the diagnosis of leptospirosis. In that group of 16 dogs, 8 dogs had convincing clinical evidence of leptospirosis, including 6 dogs with acute renal failure, 1 dog undergoing chemotherapy, and 1 dog with polyuria-polydipsia. The dog with polyuria-polydipsia had 1 of the strongest PCR bands of all dogs tested on the initial test, and clinical signs resolved within 5 days of administration of doxycycline.

Three dogs with chronic renal failure and positive results of PCR assay without confirmed leptospirosis either had low titers to serovar hardjo (n = 2) or were seronegative (1). All 9 dogs with chronic renal failure that had a negative PCR test result were seronegative (<1:100) for all serovars tested, raising the question as to whether the low titers to serovar hardjo in these 2 dogs were clinically important. It is interesting to speculate that the seronegative dog that lived with a prairie dog may have been infected with a serovar that was not serologically tested for.

Of the 5 remaining dogs with positive results of PCR assay and no confirmed leptospirosis, 4 did not have clinical disease or had another diagnosis. The 2 German Shepherd Dogs were both farm dogs and could have had exposure to leptospires in the environment, raising the question as to whether they pose a zoonotic threat without being clinically ill. Likewise, the 2 housemates that were active rodent hunters were never ill but may have posed a zoonotic threat to the pet owner. This raises the question of the importance of
the zoonotic risk from occult shedding in a healthy dog. Findings in the Newfoundland with paresis that responded to doxycycline administration remain a mystery. The dog became progressively worse during a 2- to 4-week period to the point of being nonambulatory, but responded rapidly to doxycycline administration. This clinical syndrome has not been described in canine leptospirosis, although the concurrent presence of uveitis in this dog raises the possibility of a leptospiral CNS infection. Confirmation by serologic conversion would have been convincing in this dog.

It is possible that the PCR assay may be a more sensitive diagnostic test than serologic testing that is used in practice. The low positive predictive value obtained for PCR testing in this study is predicated on the assumption that serology is the gold standard in clinical practice for confirming a diagnosis of leptospirosis. However, serologic results may not always be positive in dogs or other animals with leptospirosis. Adamus et al. identified 11 dogs from a breeding colony with chronic active hepatitis that were “unequivocally infected” with leptospires but described their serologic responses as poor and inconsistent, with only 3 of these 11 dogs having high titers to nonvaccinal serovars (autumnalis and australis). Birnbaum et al. identified 1 dog in their study that was seronegative to 5 serovars but had a positive result of a urine fluorescent antibody test. This dog was subsequently identified as being seropositive to serovar autumnalis. In a study that evaluated the detection of leptospires in bovine semen by use of PCR assay, although 80% (16/20) of the samples yielded positive results of PCR assay, only 45% (9/20) of the cattle were seropositive. Five semen samples from seronegative bulls in that study yielded positive results of PCR assay and bacteriologic culture, whereas only 2 semen samples from seropositive bulls yielded negative PCR assay and culture results. Finally, in a study that used PCR assay to detect Leptospira spp in the aqueous humor of horses with uveitis, 21 of 30 horses had positive results of PCR assay, and 6 of these 21 had positive results of culture; however, 2 horses with positive results of PCR assay and culture were seronegative. Additionally, the cultured isolates were not reactive with any of the serovars used in the microscopic agglutination test, suggesting a previously unidentified serovar. Results of these studies suggest that reliance on serologic evidence of infection alone may result in missing the diagnosis in a substantial number of animals, and that a combination of tests may be necessary. Reassessment of the sensitivity and specificity by including the 8 dogs in our study with a high suspicion of having leptospirosis in the true positive group and leaving the results from the remaining 8 dogs as false positives would result in sensitivity of 100%, specificity of 94%, positive predictive value of 67%, and negative predictive value of 100%.

There are several possible explanations for finding seronegative dogs that tested positive by PCR assay. Those dogs may have been infected by a serovar (eg, autumnalis) that was not detected serologically, immunosuppressed and unable to mount an antibody response, or died prior to seroconversions; the leptospires may have been localized in a site (eg, renal tubules) sequestered from the immune system, the dogs may have been carriers-shedders of leptospires without clinical disease, or the results may have been false positives, and no leptospiral DNA was present. Three healthy dogs in our study were PCR positive, including 2 active rodent hunters, suggesting the possibility that dogs may shed leptospires in urine without clinical illness and, therefore, may pose a risk of zoonotic infection. In a study by van den Broek et al., 3 of 7 dogs in which leptospires were isolated from the urine were seronegative, and only 1 dog had high titer to the serovar that was isolated. Dogs that shed leptospires in the urine without clinical signs may be given a misdiagnosis of leptospirosis on the basis of PCR assay results if they develop a disease that has clinical signs similar to leptospirosis and an incomplete diagnostic evaluation is performed.

References