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Exposure to *Leptospira* spp. in Sick Dogs, Shelter Dogs and Dogs from an Endemic Area: Points to Consider*

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ABSTRACT

***Background*:** Leptospirosis is a zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. Rodents play an important role as maintenance hosts, but dogs can be significant reservoirs for human infection in tropical areas as well as the source of disease outbreaks. Manifestations of disease in dogs vary from asymptomatic carriers to severe clinical signs and death. This study compared leptospiral exposure in dogs suspected to have leptospirosis and presented at a Veterinary Teaching Hospital (VTH), dogs from a Control Center of Zoonoses (CCZ) and dogs from a neighborhood with a high prevalence of human leptospirosis. Also, clinical signs, laboratory abnormalities and environmental risk factors associated with the infection were investigated at a population level and in a case-by-case approach.

***Materials, Methods & Results*:** Between May 2007 and February 2009, 253 dogs from Porto Alegre, Brazil, were enrolled in the study. Three populations were evaluated including dogs from and endemic area to human leptospirosis, dogs from a CCZ and dogs presented to a VTH. All dogs' owners from the endemic area and from the VTH answered a questionnaire including dog's information such as breed, age, vaccination status, environment, contact with other domestic animals, presence of rodents in the household, clinical signs, medications and if owners had leptospirosis diagnosed in the previous two years. The investigation of the exposure to pathogenic leptospires was based on serology using the Microscopic Agglutination Test (MAT), and polymerase chain reaction (PCR) using two sets of primers to detect pathogenic leptospiras in blood (leptospiremia) and urine (leptospiruria). Positive results were found in the three populations. The most prevalent serovars were Canicola, Icterohaemorrhagiae and Copenhageni, independent of the dog health condition. Leptospiruria occurred in 20.0%, 8.4% and 30.3% of CCZ, endemic area and VTH dogs, respectively. There was no association between seropositivity and leptospiremia or leptospiruria. The presence of rats in the environment was associated with leptospiruria ($P = 0.02$). Complete blood count (CBC), serum biochemistry (alanine aminotrasferase and creatinine) and urinalysis were also performed. Although increased serum creatinine ($P = 0.009$), jaundice ($P = 0.004$) and glucosuria ($P = 0.04$) were associated with leptospiruria in the VTH dogs, the absence of clinical signs or clinicopathologic alterations did not exclude the infection, as observed in several dogs from CCZ and from the endemic area.

***Discussion*:** As expected, the VTH showed the relatively highest percentage of positive samples (serology, leptospiruria and leptospiremia), since these were clinical cases. However, no statistical differences were found in the percentage of leptospiremia between VTH and the dogs from endemic area, neither in the percentage of leptospiruria or serology between VTH and dogs from CCZ. The most common serovars identified by MAT were consistent with the findings of other studies involving dogs in Southern Brazil. If creatinine is elevated, particularly if jaundice is present, the likelihood of leptospiral infection must be considered; however, normal findings for these parameters do not rule out this diagnosis. Most of the dogs vaccinated the year before showed leptospiremia and/or leptospiruria, suggesting infection with a serovar not included in the vaccine, vaccine inefficacy or a wrong dog vaccination schedule. Subclinical infection is a problem when considering animals with leptospiruria that will likely remain untreated. The control of the environmental dissemination of pathogenic *Leptospira* spp. in urban settings should include the identification of asymptomatic dogs.

Keywords: Leptospira, zoonoses, risk factor, diagnosis, urine.

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INTRODUCTION

As a neglected tropical disease, leptospirosis has been increasingly observed in urban settlements, especially in slums from developing countries [8]. Contaminated urine with pathogenic leptospires is the main source for dissemination of the disease. Although rodents play an important role as maintenance hosts, dogs can be significant reservoirs for human infection as well as the source of disease outbreaks [3]. Nevertheless, little has been done to evaluate the clinical presentation and overall spread of leptospirosis in dogs having close contact with humans.

Infection with pathogenic *Leptospira* spp. ranges from subclinical to a syndrome of multi-organ involvement in animals and humans. Clinical signs of canine leptospirosis depend on the age and immunological status of the host, environmental factors, and the virulence of the infecting serovar [5].

The aim of this study was to compare leptospiral exposure in three different dog populations including a Control Center of Zoonoses (CCZ), an isolated low-income neighborhood with endemic human infection, and a referral Veterinary Teaching Hospital (VTH). Also, clinical signs, laboratory abnormalities and environmental risk factors associated with the infection were investigated at a population level and in a case-by-case approach.

MATERIALS AND METHODS

Study design and sample

Between May 2007 and February 2009, three different dog populations from Porto Alegre City, Brazil, were evaluated for leptospirosis. One group consisted of owned dogs (n = 155, 71 females and 84 males) living outdoor, from a low-income, poverty line, endemic neighborhood, named Arquipélago. This is a flood area with a high population of dogs and rodents, previously reported as an endemic area for human leptospirosis [7]. Samples were collected from dogs ≥ 1 year-old after clinical examination. Other group consisted of adult dogs (n = 65, 10 females and 55 males) from a CCZ that rescues stray dogs and receives relinquished dogs; if clinically healthy and unclaimed; the dog is offered for adoption. The third group consisted of dogs (n = 33, 11 females and 22 males) presented to a VTH; the dogs from this group were suspected of acute or chronic leptospirosis based on the presence of some risk factor (rats in the environment or unvaccinated dog living outdoor) associated to two or more clinical signs (jaundice, anorexia/weight loss, vomiting, diarrhea, polyuria/polydispsia, fever) or to laboratorial alterations such as leukocytosis, high serum creatinine or high serum alanine aminotransferase (ALT). Animals receiving antibiotic therapy at the time of evaluation were excluded from the study. All dogs' owners from the endemic neighborhood and from the VTH agreed to volunteer in this study, and answered a questionnaire including dog's information such as breed, age, vaccination (vaccinated <1 year ago, vaccinated >1 year ago or unvaccinated) status, environment, contact with other domestic animals (horses, cats, cattle, pigs, etc), presence of rodents in the household, clinical signs, medications and if owners had leptospirosis diagnosed in the previous two years.

Definitive diagnosis of exposure/infection to *Leptospira* spp. was considered if the dog showed positive serology or positive PCR in blood or urine.

Urine was collected by voiding or catheterization. One aliquot was used for urinalysis, and another was immediately mixed to 1X PBS pH7.4 in order to neutralize the urine for DNA extraction [11]. DNA extraction was performed in duplicate.

Blood samples were collected in vacuum tubes with no additive and tubes containing EDTA. Serum was stored at -20°C for serological and biochemical analysis (creatinine and ALT). One aliquot of EDTA sample was used for complete blood count (CBC), and another was frozen at -20°C until DNA extraction. DNA was extracted using a commercial kit following manufacturer's protocol[1]. An internal control target, the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) [16], was performed in all DNA samples from blood and insured successful extraction.

Serology

MAT was performed to detect the presence of antibodies against 13 *Leptospira* antigens, considering titers ≥100 as positives and the highest titer when more than one serovar reacted. The tested serovars included Australis, Autumnalis, Bratislava, Canicola, Copenhageni, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pomona, Pyrogenes, Tarassovi and Wolffi.

PCR

For the detection of pathogenic leptospires in blood (leptospiremia) and urine (leptospiruria) the primer sets G1/G2 and B64-I/B64-II [4] were used to amplify a fragment of DNA from *secY* and *flaB* genes, respectively. G1 (5' CTG AAT CGC TGT ATA AAA GT 3') and G2 (5'GGA AAA CAA ATG GTC GGA AG 3') amplify DNA of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchi*, *L.santarosai*, and *L. meyeri* species, whereas B64-I (5' ACT AAC TGA GAA ACT TCT AC 3') and B64-II (5' TCC TTA AGT CGA ACC TAT GA 3') amplify DNA of *L. kirschneri*. The sensitivity testing of the singleplex PCR showed that leptospires were consistently detected (100%) at 10 copies/reaction (25µL) for primers G1/G2 and 50 copies/reaction (25 µL) for primers B64-I/B64-II; 5 and 25 copies/reaction for primers G1/G2 and B64-I/B64-II could be detected, but less consistently with a success rate of 5 /10 and 4/10 positive PCR reactions per attempts, respectively (data not shown). All the blood and urine samples were tested for both primer sets.

Statistical analysis

Association between the parameters including blood analysis, urine analysis, clinical signs and each of blood PCR, urine PCR, and serological results was done using Chi square test, or Fisher's exact when appropriate, using Stata 11 (StataCorp, College Station, TX 77845, USA). Continuous variables such as blood cells count, biochemistry parameters, and urinalysis parameters were transformed into categorical variables. Multivariable logistic regression model with forward stepwise variable selection was used to evaluate the association between each of the outcomes (blood PCR, urine PCR, and serology) and blood and urine analysis parameter, clinical signs, as well as the environmental risk factors. Goodness of fit test was used to assess the model fitness and variable selection. A P-value <0.05 was considered significant

Table 1. Microscopic agglutination test and PCR in blood and urine to detect leptospiral exposure/infection in shelter dogs, dogs from an endemic area and sick dogs in Porto Alegre City, Brazil.

Dogs	Positive PCR in blood	Positive PCR in urine	Positive serology
CCZ (n=65)	5[a] (7.7%)	13[c] (20.0%)	35[e] (53.8%)
Endemic area (n=155)	23[a,b] (14.8%)	13[d] (8.4%)	63[f] (40.6%)
Veterinary Hospital (n=33)	9[b] (27.3%)	10[c] (30.3%)	24[e] (72.7%)
Total (n=253)	37 (14.6%)	36 (14.2%)	122 (48.2%)

Same letters means no difference between groups.

RESULTS

Leptospiral exposure

Results of PCR and serology are shown in Table 1. Positive serology and PCR were observed in all three groups. VTH group had the highest prevalence of positive cases, based both in serology and PCR. The endemic area had the lowest number of positive PCR in urines. Five (2%) of all dogs had concurrent leptospiremia and leptospiruria (both PCR positives). Most of the positive PCR results (65/73) were detected using primers G1/G2. Using primer set B64-I/B64II, six blood and two urine samples from the endemic area were positive, demonstrating the presence of the species *L. kirschneri* in this population only. Four of these dogs that showed leptospiremia had clinical signs such as vomiting, diarrhea, anorexia, and polyuria/polydipsia, whereas the two dogs with leptospiruria were clinically unaffected.

A total of 48.2% (122/253) of the sera samples reacted with one or more serovars. All 13 serovars tested on MAT reacted at least once. The identification of a predominant serovar was possible in 57/122 samples (higher titer or reactive to one serovar only), being Canicola (13/57), Icterohaemorrhagiae (11/57) and Copenhageni (10/57) the most prevalent. Titers ≥ 200 (200 to 1,600) were detected in four dogs only, including two sera against serovar Canicola and two against serovar Copenhageni. Three of these dogs tested negative by PCR in blood and urine, however the dog having a titer 1,600 (serovar Copenhageni) was positive by PCR (G1/G2 primers) in urine.

There was no association between seropositivity and positive PCR in blood or urine. Only 34.4% of seropositive dogs had positive PCR in blood and/or urine. On the other hand, 10.3% dogs that had leptospiremia and/or leptospiruria were negative on MAT. In multivariable logistic regression analysis for serology, none of the indicator variables were significant (data not shown).

Risk factors

With the exception of breed and gender, which were evaluated in all groups, risk factors identified by questionnaire responses were only analyzed in the endemic area and VTH populations. There were no associations between positive PCR or positive serology and breed, gender or age.

None of the dogs from the endemic area received vaccine in the year before sampling. Only six dogs were vaccinated less than one year before presenting to the VTH; one of these dogs had leptospiremia, three had leptospiruria and one had concurrent leptospiremia and leptospiruria. There was a significant association between positive serology and dogs vaccinated within the last year, in Veterinary Hospital group ($P = 0.047$). From the six vaccinated dogs, two had cross-reation in MAT, one had titer 100 to serovar Pomona, one had titer 100 to serovar Autumnalis, and two had negative serology.

Contact with other domestic animals was not associated to positive serology or PCR in the studied populations. The presence of rats in the environment was associated with positive PCR in urine ($P = 0.02$). All dogs from the endemic area had direct or indirect contact with rats, i.e., presence of these rodents in the household (in fact, some of these animals were defined as rat hunters by their owners). Five dog's owners from the endemic area had a previous diagnosis of leptospirosis, but their dogs had no leptospiruria at the time this study was carried out. Nevertheless, two of these dogs had leptospiremia.

Clinical and clinicopathologic findings

Jaundice occurred in VTH dogs only, and an association between positive urine PCR and jaundice was observed in this group ($P = 0.004$). Furthermore, icteric dogs had 21 times the odds of having leptospiruria (PCR urine positive) comparing to non-icteric dogs. Although 24.6% and 22.6% of the dogs from CCZ and from the endemic area, respectively, showed positive PCR in blood and/or urine, none of them were icteric. Using Chi-square test, an association between diarrhea and positive serology ($P = 0.044$) was found, but not between diarrhea and leptospiremia or leptospiruria. However, when multivariable logistic regression model was evaluated, dogs with diarrhea had 4.67 times the odds of having leptospiremia when compared to dogs having no diarrhea. Other clinical signs of dogs from the endemic area and VTH were not significantly associated with the leptospiremia or leptospiruria.

No association between anemia or other CBC alterations (total and differential leukocyte count, red blood cells parameters) and positive PCR or serology was observed, although dogs with leukocytosis had 2.61 times the odds of having leptospiremia compared to dogs having normal leukocytes values. Serum creatinine values above the normal range occurred in VTH only, and were associated with leptospiruria ($P = 0.0091$) and with positive serology ($P = 0.0002$), but not with leptospiremia. High serum ALT was not associated with positive PCR or serology results. The VTH group showed a significant association between leptospiruria and glucosuria ($P = 0.04$).

DISCUSSION

Although it is known that *Leptospira* spp. is widely disseminated in the world, the true frequency of affected dogs is probably still underestimated due to the large number of asymptomatic animals. In this study, dogs shedding leptospires were detected in all groups, but dogs from CCZ and dogs from the endemic area, which were mostly apparently healthy, are more likely to play an important role in public health than the sick dogs for which veterinary care is sought. When considering animals from the CCZ and endemic area, no attempts are made to avoid the environmental contamination with infected urine or to treat infected animals, especially because these dogs show no clinical signs of the disease. Thus, further investigations should be performed to evaluate the role of the asymptomatic dog as a *Leptospira* spp. disseminator and/or potential direct source of human infection, especially in low-income urban settings.

PCR assays are especially useful for early diagnosis of leptospirosis and to detect chronic carriers [19]. The detection of antibodies by MAT has been historically applied for diagnosis of leptospirosis and is a useful tool for epidemiological studies [2]. The high-

est number of dogs diagnosed with positive serology or PCR observed in the VTH population was expected, since these dogs had been included in the study due to the presence of clinical and/or laboratorial alterations compatible with leptospirosis [2]. However, some dogs of VTH group tested negative for MAT or PCR, showing the importance of investigating other causes for the sickness and abnormal clinicopathologic findings in such cases. Conversely, although clinical signs and hematological, urinary and biochemistry alterations cannot be used to predict leptospirosis, it is very useful to monitoring the treatment and prognosis of the patient. In CCZ dogs, the percentage of dogs with leptospiruria was higher than leptospiremia. Although this may reflect the leptospiral exposure picture of stray dogs in the city, it is also possible for the infection to have been acquired at CCZ as multiple dogs were housed in each pen. It was somewhat surprising that the prevalence of leptospiremia was higher than the prevalence of leptospiruria in the dogs of the human endemic area. However, there are several plausible explanations for this finding. A recent outbreak of leptospirosis on this area is one possibility, however the authors also speculate that different serovars that are less likely to localize to the kidney may infect these dogs. The lower number of seropositive dogs on the endemic area in comparison to the CCZ and VTH populations also may be explained by the presence of different serovars on the neighborhood, which were not detected by the routine serologic testing [2]

Despite the importance of serology for epidemiological evaluation, the interpretation of the results might be done with caution; titers indicated antibodies production, but even single titers ≥800 do not confirm infection [18]. The most common serovars identified herein were consistent with the findings of other studies involving dogs in Rio Grande do Sul State [12,13,15]. The positive association between serology and vaccine in the VTH group could be related to vaccine titer [5], but the leptospiremia and/or leptospiruria found in 5/6 of these vaccinated dogs suggests infection with serovars different from those present in the vaccine, vaccine inefficacy or a wrong dog vaccination schedule.

The presence of positive PCR in blood and/or urine in 26 dogs of this study in absence of positive MAT emphasize that serology may not be used alone to exclude the infection. Serology has been reported as a poor predictor of urinary shedding of leptospires in dogs [6]. Negative serology in the presence of positive PCR could be attributed to an early stage of the disease [14], a serovar not included in the panel of tested MAT [1], or to lower titers (less than lowest MAT titer) of antibodies in the chronic stage; with these animals becoming seronegative carriers [10]. Dogs are well adapted to serovar Canicola and may actively shed leptospires with titers under 100 [9]; we hypothesized that this event could have occurred with some seronegative dogs with leptospiruria herein, since this serovar was one of the most prevalent in this study.

The species *L. kirschneri* was found only in dogs from the endemic area. Although the serogroup Grippotyphosa is the most common cause of disease in dogs in the United States [17,20] there are nine other serogroups of the species *L. kirschneri* [10] that could have given a positive PCR in this study. Voles, raccoons, skunks and opossums are implicated as maintenance hosts of serovars Grippotyphosa in several countries, but the hosts that may serve as reservoirs in Brazil have not been defined yet. Some of the dogs on the endemic area with leptospiremia caused by *L. kirschneri* had clinical signs, suggesting that they are probably incidental hosts of the infective serovar.

In the endemic area, some people had leptospirosis previously, and some of their dogs had leptospiremia but not leptospiruria at the time of this study. It suggests that these dogs were recently infected and were not the source of the previous owners' infection. However, re-infection and elimination cannot be excluded in these cases, and the role of the dog as a direct source for human infection cannot be ruled out.

It is important to emphasize that apparently healthy dogs may have leptospiremia or be a silent carrier as observed in several dogs from the endemic area and CCZ. If creatinine is elevated, particularly in an icteric dog, the likelihood of leptospirosis must be considered; however, normal findings for these parameters do not rule out this diagnosis. *L. kirschneri* infection was detected by PCR in dogs from the endemic area. Whether or not human infection with *L. kirschneri* is occurring, and the possibility that dogs serve as a reservoir for such infections, need to be investigated. Further, the use of an effective vaccine that includes serovars of *L. kirschneri* infecting dogs on the endemic area is indicated. Nevertheless, we hypothesize that the control of the environmental dissemination of pathogenic *Leptospira* spp. in urban settings should include the evaluation of the presence of asymptomatic dogs.

SOURCE AND MANUFACTURER

[1]QIAamp DNA Blood Mini kit, QIAGEN, Valencia, CA, USA.

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***Ethical approval*.** The present study has been submitted and approved by the University Ethics Committee, following ethical principles for animal experimentation of the Brazilian College of Animal Experimentation.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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