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Short Communication

Serological and molecular detection of *Leptospira* spp in dogs

Giulia Soares Latosinski[1], *Felipe Fornazari*[1], *Selene Daniela Babboni*[1], *Karen Caffaro*[1], *Antonio Carlos Paes*[1] *and Helio Langoni*[1]

[1]. Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista *Júlio de Mesquita Filho*, Botucatu, SP, Brasil.

Abstract

Introduction: This study aimed to detect anti-*Leptospira* spp antibodies and *Leptospira* DNA in domestic dogs. **Methods:** Blood and urine from 106 dogs were evaluated by microscopic agglutination test (MAT) and polymerase chain reaction (PCR), respectively. **Results:** Six (5.7%) and one (1%) animals were positive by MAT and PCR, respectively. **Conclusions:** These results show a low prevalence of infection by *Leptospira* spp. The absence of positive results for the Icterohaemorrhagiae serogroup indicates the small relevance of these dogs as sources of human leptospirosis.

Keywords: Leptospirosis. PCR. Microscopic. Agglutination test.

Leptospirosis is a zoonotic disease that affects humans and domestic and wild animals through direct or indirect contact with the urine of infected hosts, mainly rodents[1]. Environmental risk factors contribute to the endemic character of the disease, especially in developing countries. In Brazil, poor sanitary conditions are common, including untreated sewage and garbage accumulation, which predisposes the proliferation of rodents and thus may expose humans, dogs, and other animals to leptospirosis[2].

Leptospirosis is considered an emerging disease due to its increased incidence among populations such as domestic dogs in some regions of the world[3]. This change may be associated with climate change, which favors the increased survival of leptospires in the environment[2]. Tropical climate, standing water, poor sanitation, and proximity to animal reservoirs intensify the epidemic character of leptospirosis in developing countries[2]. Besides rodents, dogs can also play an important role in the epidemiology, acting as accidental or maintenance hosts. Dogs can also be sentinels for several diseases, assisting in pathogen detection in a particular area[4].

The gold-standard serological test for leptospirosis is the modified agglutination test (MAT), which has a specificity and can be used to identify the infecting serogroup of the bacterium, thus helping to detect the probable animal source of infection[1]. Polymerase chain reaction (PCR) is another important diagnostic test, which detects leptospiral deoxyribonucleic acid (DNA) in several types of samples, including blood, urine, semen, and organs. Compared to bacterial culture, PCR is faster, more specific, and sensitive[5].

The present study aimed to evaluate the role of dogs in the epidemiology of human leptospirosis in Botucatu county, São Paulo, Brazil, through the detection of anti-*Leptospira* antibodies and leptospiral DNA in dogs. According to the serogroups identified by MAT and the frequency of animals shedding leptospiral DNA, we can propose if dogs may be involved in the epidemiology of human leptospirosis.

Blood and urine samples were collected from 106 asymptomatic dogs in Botucatu County, São Paulo State, Brazil, which has an estimated dog population of 26,721 animals and a prevalence of anti-*Leptospira* antibodies ranging from 15 to 20% in asymptomatic dogs, according to previous investigations[6,7]. The study was conducted between October 2014 and June 2015.

Samples were collected during the municipal vaccination campaign against rabies in the Municipal Kennel of Botucatu and during medical care at the Veterinary Hospital from the School of Veterinary Medicine and Animal *Science* [*Faculdade de Medicina Veterinária e Zootecnia* (*FMVZ*)], *São Paulo State University*, Botucatu [*Universidade Estadual Paulista Júlio de Mesquita Filho* (UNESP)]. All samples were collected with consent of the dog's owner. Phosphate buffered saline solution (PBS) pH 7.6 was added to urine in a 1:1 proportion and the tubes were frozen at -20°C. Most of the dogs included in the study were stray animals taken to the municipal kennel for castration. Thus, epidemiological data were lacking.

Detection of antibodies was performed using the MAT. A collection of 12 antigens maintained at 28°C in Ellinghausen-McCullough-Johnson-Harris media (EMJH), was used: Australis,

Corresponding author: Prof. Helio Langoni
e-mail: hlangoni@fmvz.unesp.br
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Bratislava, Autumnalis, Canicola, Sentot, Grippotyphosa, Copenhageni, Icterohaemorraghiae, Pomona, Pyrogenes, and Hardjo. The results were presented at the serogroup level as recommended by Sykes et al.[8], adopting 100 as the cut-off. The MAT was performed as previously described by Fornazari et al.[5]. PCR was used to detect leptospiral DNA. First, the limit of detection (LOD) of the assay was determined. Serial dilutions of an antigen for the MAT (serovar Hardjo) were made in PBS and bacterial count was assessed by dark field microscopy using a Neubauer chamber. Each dilution was tested by the PCR protocol described below, which resulted in an LOD of 10 bacteria/µL.

Urine samples were thawed and washed twice with sterile PBS prior to DNA extraction. One milliliter of each sample was centrifuged in DNAse/RNAse free microtubes at 11,000× *g* for 10 minutes. The supernatant was removed and the pellet was resuspended in 1mL of PBS and homogenized in an automatic agitator (IKA®). The sample was centrifuged again and the same washing procedure was repeated. The pellet was resuspended in a final volume of 200µL of PBS.

DNA extraction was performed using the Illusta™ Blood Genomic Prep Mini Spin Kit (GE Healthcare®) according to the manufacturer's instructions. PCR was carried out in 200µL microtubes containing 3µL of sample, 12.5µL of GoTaq® Green Master Mix (Promega), 1µL of each primer (10ρmol/µL) and 7.5µL of Milli-Q water, for a total volume of 25µL per reaction. We used the Lep1 and Lep2 primers described by Merien et al[9], which corresponds to oligonucleotides 38-57 (5'GGCGGCGCGTCTTAAACATG3') and 348-368 (5'TTCCCCCCAT TGAGCAAGATT3') of the 16S rRNA gene from *Leptospira interrogans*, resulting 331-base pair product. Milli-Q water and an antigen kept at EMJH (serovar Hardjo) were used as negative and positive controls, respectively.

DNA amplification was performed in a thermocycler (Matercycle ep Gradient, Eppendorf) using the following conditions: initial denaturation at 94°C for 3 minutes, 30 cycles at 94°C for 1 minute, annealing at 63°C, and a final step for DNA extension of 72°C for 2 minutes. The PCR products were submitted to horizontal electrophoresis in agarose gel (1.5%) stained with Nancy-520 (Sigma®) and the bands were visualized using a GelDoc-ItTM Imaging System.

Six animals were positive by MAT (5.7%; IC 95% 2.2 - 10%) and all reacted to more than one serovar. Only the serovar with the highest titer was considered the probable agent that caused infection. The positive results to the remaining serovars were considered cross-reactions between different antigens. In one sample the same titer was observed for two serovars; this animal was considered positive for both. Canicola was the most common serogroup (66.6%), followed by Autumnalis (33.3%) and Grippotyphosa (16.6%). The antibody titers ranged from 200 to 1,600. The results are summarized in **Table 1**. Only one animal was positive by PCR (1.0%; IC 95% 0.0 - 2.7%), which was negative by MAT.

We observed a low prevalence of dogs positive for *Leptospira* spp infection in the region of Botucatu. Previous studies have demonstrated that the prevalence in dogs can range from 7 to 32%[10, 11]. These differences can be explained by several factors, including temperature, animal reservoirs, topography, rainfall, and many other environmental features.

Similar studies conducted in Botucatu reported a higher prevalence, such as 17.9%[6] and 15.3%[7]. Our results may suggest that the prevalence of *Leptospira* spp infection in dogs has decreased in Botucatu, although this hypothesis cannot be confirmed using these data. Another important factor is the sensitivity of MAT. Tulsiani et al.[12] stated that the antigens used in the MAT can become attenuated with successive passages in vitro, which can reduce the sensitivity of this test. This could have occurred in our antigens collection with long-term maintenance *in vitro*. However, the low positivity of MAT was corroborated by the PCR results, which also indicated a low prevalence. Therefore, it seems improbable that the seroprevalence was influenced by a biased methodology.

Dogs are considered a maintenance host of serovar Canicola, presenting with subclinical infections or acute clinical disease; in both cases, the dogs can become renal carriers of leptospires[13]. This serogroup was the most prevalent in our study, consistent with data from previous studies[11]. Although serogroups Autumnalis and Grippotyphosa have also been reported in dogs, they were detected in only two animals. According to Hagiwara et al.[13], dogs from different regions of Brazil are infected by Canicola and Pyrogenes serogroups; Autumnalis and Gryppotyphosa are also observed but at a

TABLE 1: Results of the MAT in sera samples of dogs from Botucatu County, São Paulo State, Brazil.

Serogroup	Titers						
	100	200	400	800	1,600	3,200	Total
Canicola	-	1	1	1	-	-	3
Autumnalis	-	-	-	1	-	-	1
Grippotyphosa	-	-	-	-	1	-	1
Autumanalis + Canicola	-	-	1	-	-	-	1
Total	-	1	2	2	1	-	6

MAT: microscopic agglutination test.

lower prevalence. None of the dogs in our study were positive for the Icterohaemorrhagiae serogroup, which is the most important in public health. Therefore, it is unlikely that these animals play a major role in the epidemiology of human leptospirosis in the region of Botucatu. A recent study reported a high prevalence of dogs positive for serogroup Icterohaemorrhagiae in PCR of urine sample[14]. This study was conducted in a leptospirosis-endemic area, which probably explains the difference in relation to our results since Botucatu is a non-endemic city. Between 2010 and 2015, only nine cases of human leptospirosis were notified according to Brazil's Information System for Notifiable Diseases. Researchers have debated if dogs are really relevant in the transmission of leptospirosis to humans[15] and, until now, there has been no consistent data on this fact.

The antibody titers ranged from 200 to 1,600 and three animals had titers equal to or higher than 800. The cut-off of 800 is considered indicative of clinical disease in humans. However, there are not currently any specific standard criteria for dogs. All dogs in this study were apparently healthy, indicating that high titers are not always associated with disease symptoms. This finding is corroborated by our personal experience as well as by the literature[16]. Thus, it is important to associate laboratory diagnosis with clinical history and epidemiology so MAT results can be interpreted properly.

PCR allowed us to assess the potential of dogs as carriers of leptospires. Few studies using PCR in dogs have been performed, especially in Brazil. Our results indicated a low frequency of dogs carrying leptospires in the urine. Bacterial shedding is intermittent; thus, sampling animals more than once could indicate a higher prevalence. However, in this case, we believe that the number of dogs positive by PCR would still be low, corroborating the low frequency of positive animals by MAT. It is also possible that the PCR results were associated with small concentrations of leptospires in urine, which can be below the PCR detection threshold when animals are chronically infected by adapted serovars.

The dog positive by PCR was negative by MAT, a result that can be explained by the pathogenesis of leptospirosis. Antibodies can be detected 10 to 14 days after infection, with high levels between 21 and 42 days that can be maintained for six weeks. A gradual reduction occurs until titers are low titers (or undetectable). Leptospiruria starts 14 days after infection, is intermittent, and can last for just a few days or more than two years[1]. In addition, the *Leptospira* spp detected by PCR could belong to a serogroup that was not included in the MAT. Disagreement between MAT and PCR is common and has been observed in many studies regardless of animal species[5,14].

One of the limitations of this study was the low sample size as it was not representative of the dog population in Botucatu. We did not sample more animals because this preliminary investigation focused mainly on the role of dogs as carriers of leptospires. In addition, the narrow confidence interval of the PCR results (0-2.7) indicates the accuracy of these data.

In conclusion, these results contribute to the understanding of leptospirosis epidemiology in the study region. The investigated dogs had a low prevalence of infection by *Leptospira* spp, with a higher positivity for Canicola serogroup. The absence of positivity for the Icterohaemorrhagiae serogroup suggests that these dogs are not involved in the epidemiology of human leptospirosis. This hypothesis is reinforced by the low frequency of dogs shedding leptospires. However, the detection of just one animal positive by PCR could have significant implications for environmental contamination depending on the pathogenicity of the leptospires, bacterial load in urine, its survival in the environment, and the shedding period. More studies are needed to address these questions.

Conflict of interest

The authors declare that there is no conflict of interest.

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