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Study of urinary shedding and identification of chronic carriers of pathogenic leptospires in dogs kept in public or private animal shelters of metropolitan São Paulo area

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RESUMO

MIOTTO, B. A. **Avaliação da leptospirúria e identificação de portadores de leptospiras patogênicas em cães mantidos em abrigos públicos ou particulares da região metropolitana de São Paulo.** [Study of urinary shedding and identification of chronic carriers of pathogenic leptospires in dogs kept in public or private animal shelters of metropolitan São Paulo area]**.** 2016. 104 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

A leptospirose é uma doença zoonótica de importância global causada por espécies patogênicas do gênero *Leptospira*. Cães são hospedeiros de manutenção de leptospiras patogênicas e podem atuar como potenciais fontes de infecção da doença. A identificação de tais indivíduos e a caracterização de leptospiras envolvidas na infecção crônica podem ajudar a compreender o papel dos cães na epidemiologia da doença tanto em ambientes rurais quanto urbanos. O presente trabalho descreve a identificação de cães errantes e mantidos em abrigos coletivos com eliminação assintomática de leptospiras patogênicas, além de descrever também a caracterização das diferentes estirpes obtidas de cães cronicamente infectados. Amostras de sangue e urina foram coletadas de 3 populações distintas: (I) 92 cães mantidos em um abrigo coletivo localizado dentro da Universidade de São Paulo; (II) sete cães errantes capturados dentro do campus da Universidade de São Paulo; e (III) 24 cães mantidos em um abrigo coletivo localizado na cidade de Mogi das Cruzes. Cães identificados como leptospirúricos por técnicas moleculares (PCR) foram prospectivamente avaliados para confirmar a persistência da eliminação bacteriana e para obter isolamento da cepa infectante e sua subsequente caracterização. A amplificação de fragmentos dos genes 16S rRNA e *lipL32* permitiu a identificação de 10 cães (10,87%) leptospirúricos na população I. Dois dos 10 cães haviam sido recentemente admitidos no local, e outro cão foi adotado logo após apresentar grandes quantidades de leptospiras na urina. A avaliação prospectiva de nove animais leptospirúricos permitiu a caracterização da infecção crônica e assintomática em dois cães, o que possibilitou o isolamento de leptospiras de ambos os animais. As cepas foram tipificadas pelas técnicas de MLST e sorogrupagem, caracterizando duas cepas distintas, sendo elas *L. interrogans* sorogrupo Canicola e *L. santarosai* sorogrupo Sejroe. Dois cães leptospirúricos (28,5%) foram identificados na população II pela amplificação por PCR dos genes 16S rRNA e *secY*; um deles apresentou eliminação persistente de *L. interrogans*, no entanto não foi possível o isolamento do patógeno. O outro cão leptospirúrico não pôde ser reavaliado, entretanto a análise filogenética permitiu identificar infecção causada por *L. santarosai*. Apenas um cão da população III (4,1%) apresentou eliminação de leptospiras na urina, que foi confirmada pela amplificação de fragmento dos genes 16S rRNA e *secY*; o cão não pôde ser reavaliado, no entanto a

análise filogenética dos fragmentos amplificados confirmou infecção causada por *L. santarosai*. Os resultados indicam o primeiro registro de infecção causada por *L. santarosai* em cães. A ocorrência da infecção assintomática causada por essa espécie nas três populações avaliadas indica um possível papel dos cães na cadeia de transmissão desse patógeno em centros urbanos, além de demonstrar que cães podem se tornar portadores de diferentes espécies de leptospiras. Os resultados sugerem uma possível distinção genotípica de cepas de *L. santarosai* mantidas por cães quando comparadas com estirpes desta espécie isoladas de outros hospedeiros. O presente estudo também foi capaz de demonstrar que cães leptospirúricos podem ser inadvertidamente admitidos ou adotados em abrigos coletivos, aumentando potencialmente os riscos de transmissão ocupacional e zoonótica da doença.

Palavras-chave: Leptospirose. Cão. Assintomático. PCR. *Leptospira santarosai*.

ABSTRACT

MIOTTO, B. A. **Study of urinary shedding and identification of chronic carriers of pathogenic leptospires in dogs kept in public or private animal shelters of metropolitan São Paulo area.** [Avaliação da leptospirúria e identificação de portadores de leptospiras patogênicas em cães mantidos em abrigos públicos ou particulares da região metropolitana de São Paulo]. 2016. 104 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

Leptospirosis is a zoonotic disease of global importance caused by pathogenic *Leptospira* species. Dogs are reservoir hosts for pathogenic *Leptospira* and can act as potential transmission sources of the disease. Identification of such individuals and characterization of leptospires involved in chronic infections may promote a better understanding of the role of dogs in the epidemiology of particular leptospiral strains and the overall contribution of dogs to environmental contamination in urban and rural scenarios. The present work describes the identification of dogs presenting asymptomatic urinary shedding of different pathogenic *Leptospira* species among stray and sheltered dog populations, as well as the characterization of leptospiral strains isolated from chronic carriers. Blood and urine samples were taken from three different populations: (I) 92 dogs kept in a public shelter at the University of São Paulo campus; (II) seven stray dogs living inside the University of São Paulo campus; and (III) 24 dogs kept in a public shelter from the city of Mogi das Cruzes. Dogs identified as urinary shedders by PCR-based DNA detection were prospectively evaluated in order to confirm persistent renal carriage of the pathogen and to recover viable leptospires for proper characterization. Leptospiruric dogs were identified in all populations studied. Quantitative PCR targeting the *lipL32* gene and the 16S rRNA detected urinary shedding in 10 dogs (10,87%) from population I: two of these dogs were recently admitted at the facility and one dog was adopted immediately after presenting large quantities of leptospires in urine. Prospective evaluation of nine leptospiruric dogs enabled the identification of two chronic carriers, allowing the recovery of leptospires from both dogs. The strains were further characterized by MLST analysis and serogrouping, thus confirming infection caused by *L. interrogans* serogroup Canicola and *L. santarosai* serogroup Sejroe. Two leptospiruric dogs (28,5%) were detected in population II by 16S and *secY* PCR amplification; one dog presented persistent urinary shedding of *L. interrogans*, but no isolates could be recovered. The other leptospiruric dog presented asymptomatic infection caused by *L. santarosai* and could not be reevaluated. Only one dog from population III (4,1%) presented leptospiruria detected by PCR; the dog could not be reevaluated, however sequence analysis revealed infection caused by *L. santarosai*. The results indicate the first report of *L. santarosai*

infection in dogs. Asymptomatic infection caused by this leptospiral species was observed in all populations studied, thus indicating a possible role of dogs in the chain of transmission of this particular pathogen. The results also suggest a possible genetic distinction between lineages of Brazilian *L. santarosai* maintained by dogs and other animal hosts. Isolation and persistent chronic carriage of *L. santarosai* found shows that dogs can persistently harbor leptospires other than *L. interrogans*. This study also points out that dogs can be inadvertently admitted and adopted in dog shelters, potentially increasing the risks of occupational and zoonotic transmission by bringing infected animals closer to shelter workers, adopters and their households.

Keywords: Leptospirosis. Dog. Asymptomatic. PCR. *Leptospira santarosai*.

1 INTRODUCTION

Leptospirosis is a bacterial disease caused by pathogenic helical shaped motile spirochetes of the genus *Leptospira* (MOHAMMED et al., 2011). Pathogenic *Leptospira* are currently classified into more than 250 serovars, clustered into 24 antigenically related serogroups (NALAM et al., 2010). Serovar classification is based on the pattern of crossagglutinin absorption test reactivity (CAAT) against structural heterogeneity of leptospiral lipopolysaccharide surface molecules (LPS) (CERQUEIRA; PICARDEAU, 2009). However, leptospires can also be classified based on their DNA relatedness, leading to the current identification of 10 pathogenic genomospecies (BOURHY et al., 2014), including *L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L, alexanderi, L. kmetyi, L. alstonii* and *L. mayottensis.*

Leptospiral infection occurs through indirect contact of mucosal surfaces or abraded skin with contaminated soil or water or directly by contacting contaminated urine and tissues from infected animals (MOHAMMED et al., 2011). Virtually any mammalian species can be affected, causing a broad spectrum of clinical manifestations, ranging from severe life-threatening conditions and mild self-limiting febrile illness to asymptomatic infections (MOHAMMED et al., 2011).

Even though it is recognised as the most widespread zoonosis, with a major public health impact in much of the developing world, leptospirosis remains a neglected disease (BARRAGAN et al., 2016). Human leptospirosis is frequently observed in poverty-stricken populations living in tropical regions (PICARDEAU et al., 2014), and it is considered one of the major neglected diseases in Latin America (HOTEZ et al., 2008). The annual global incidence is estimated to be around 1.03 million people, with 58.900 deaths per year (ABELA-RIDDER; SIKKEMA; HARTSKEERL, 2010; SCHNEIDER et al., 2015). In Brazil, approximately 10,000 human cases are reported annually with overall case fatality of 10% among reported cases (FELZEMBURGH et al., 2014). However, unreported cases, poor medical support, inadequate access to diagnostic tests and the unspecific clinical manifestations of leptospirosis may contribute to underestimate the real burden of the disease (COSTA et al., 2015).

Environmental factors, such as natural disasters, high pluviometric precipitation rates, flooding, poor sanitary and housing conditions and close contact with livestock, companion animals and wildlife potentially exposes humans to contaminated soil and water resources (SCHNEIDER et al., 2015). Environmental contamination is mostly promoted by animal reservoirs, which can harbor leptospires in renal tubules without overt clinical signs (BARRAGAN et al., 2016). These so-called maintenance hosts present intermittent, long-lasting and highly intense urinary shedding of leptospires (MONAHAN; CALLANAN; NALLY, 2009), and the diversity of host species represents a significant challenge for disease prevention.

Rodents are considered the major source of human infection, a role likely attributed to its synanthropic behaviour and widespread distribution (COSTA et al., 2015). However, recent "One Health" approaches have been used to circumvent pivotal epidemiological aspects of leptospirosis, and several studies have pinpointed a significant role of different mammalian reservoirs in its zoonotic transmission (BARRAGAN et al., 2016; GUERNIER et al., 2016; LAU et al., 2016). The identification and management of such individuals poses as a key strategy to supplement better control programs regarding transmission between humans and animals.

Canine leptospirosis has been largely described worldwide (AZOCAR-AEDO, 2015; SCHULLER et al., 2015), and clinical presentation is often associated to *L. interrogans* and *L. kirschneri* infection (SCHULLER et al., 2015). Chronically infected animals can persistently harbor leptospires without overt clinical signs and dogs are referred as reservoir hosts for pathogenic *Leptospira* (HARKIN; ROSHTO; SULLIVAN, 2003; ROJAS et al., 2010; ZAKERI et al., 2010; GAY; SOUPÉ-GILBERT; GOARANT, 2014), notably *L. interrogans* serovar Canicola (BRANGER et al., 2005), a pathogenic serovar that can accidentally infect humans and other animals (GOULD, 1979; TREVEJO et al., 1998; WANG et al., 2015).

Despite dogs live in close contact with humans and share a considerable part of their environment, the precise role of dogs in the epidemiology of human leptospirosis remains controversial (BARMETTLER et al., 2011; MARTINS; PENNA; LILENBAUM, 2012). Previous reports addressing zoonotic transmission from dogs frequently present circumstantial evidences, mostly based on seroreactivity profile analogies (NETO et al., 1963; FRASER et al., 1973; CLEGG; HEATH, 1975; CALDAS; SAMPAIO, 1979; SCHMIDT; WINN; KEEFE, 1989; BROD et al., 2005). However, few studies have established less anecdotal associations between

canine and human leptospirosis. Feigin *et al.* reported a small outbreak compromising humans living at the same house most likely transmitted by an immunized dog carrying *L. interrogans* serovar Icterohaemorrhagiae inside their household (FEIGIN, R. D.; LOBES, L. A.; ANDERSON, D., 1973). Similarly, an epidemiological study conducted by Trevejo *et al.* has attributed dogs as potential sources of a human disease outbreak in Nicaragua (TREVEJO et al., 1998). High seroprevalence against serovar Canicola among human patients was observed, and ownership of seropositive dogs was associated with illness in humans. Moreover, the authors were able to recover *L. interrogans* serovar Canicola from six asymptomatic household dogs and two suspected human cases, suggesting dogs as an epidemiological link between environment, wild fauna and humans (TREVEJO et al., 1998). More recently, Zakeri *et al.* has found 33/150 dogs presenting urinary shedding of leptospires in Iran, with most dogs (n=29) presenting *L. wolffii* infection (ZAKERI et al., 2010). Interestingly, the same study has also found *L. wolffii* as the causative pathogen of several human cases of leptospirosis, indicating asymptomatic dogs as possible sources to human infection.

While the actual role of dogs in zoonotic transmission still remains poorly documented and the overall contribution of dogs to the burden of human leptospirosis is yet to be determined, asymptomatic urinary shedding of leptospires among dog populations has been largely reported worldwide (HARKIN et al., 2003; KHORAMI et al., 2009; ROJAS et al., 2010; ZAKERI et al., 2010; SAMIR et al., 2015; HARKIN; HAYS, 2016; LLEWELLYN et al., 2016), thus indicating that dogs at very least can contribute to the spread of pathogenic *Leptospira* strains into the environment.

Although proper management of chronically infected dogs must be implemented to reduce environmental contamination, the identification of such individuals can be challenging. Renal carriage of leptospires is not necessarily associated to the presence of serum antibodies against *Leptospira* (ANDRE-FONTAINE, 2006), limiting the use of serological tests to identify asymptomatically infected dogs. Isolation of leptospires, besides being essential to confirm infection, is also not a suitable technique for identification of urinary shedders, especially for presenting frequent contamination, fastidious growth of the pathogen and low sensitivity (SCHULLER et al., 2015). Alternatively, PCR has been successfully used to characterize leptospiruric dogs (ZAKERI et al., 2010). Nevertheless, most reports are cross-sectional studies, restricting any considerations regarding the occasional,

intermittent or persistent urinary shedding of the pathogen. The use of PCR to identify leptospiruric dogs followed by further prospective evaluation of the infected animals can provide clinical, laboratorial and serological data in order to fully characterize the dog's carrier status. More importantly, it can also potentially increase the chances of recovering leptospires in culture media for appropriate characterization. Identification of leptospiral strains remains a crucial bottleneck to determine epidemiogical aspects surrounding animal and human leptospirosis and may promote improved control and prevention strategies.

Although dogs are natural reservoirs for *L. interrogans* serovar Canicola (SCANZIANI et al., 1995), several different pathogenic strains were also recovered from asymptomatic dogs, highlighting that the classical association between particular serovars with specific maintenance hosts may not be absolute. Previous studies have shown that asymptomatic dogs might shed different *L. interrogans* serovars, such as Copenhageni and Icterohaemorrhagiae (CLEGG; HEATH, 1975; THIERMANN, 1980; GAY; SOUPÉ-GILBERT; GOARANT, 2014; SAMIR et al., 2015; SUEPAUL et al., 2015; WANG et al., 2015), Pomona (MACKINTOSH; BLACKMORE; MARSHALL, 1980; ROMERO; YASUDA, 2006; GAY; SOUPÉ-GILBERT; GOARANT, 2014), Tarassovi (MACKINTOSH; BLACKMORE; MARSHALL, 1980) and Sejroe (SCANZIANI et al., 1995; RÜHL-FEHLERT et al., 2000), all potentially pathogenic to humans. Asymptomatic infection caused by other *Leptospira* species, such as *L. kirschneri*, *L. borgpetersenii* and *L. wolffii* were also reported (CAI et al., 2002; DA CUNHA et al., 2016; HARKIN; HAYS, 2016; LLEWELLYN et al., 2016), reinforcing the unspecific nature between host-pathogen interactions and evidencing that zoonotic transmission from dogs cannot be accessed exclusively by isolating *L. interrogans* serovar Canicola from human subjects.

Renal carriage of such a repertoire of pathogenic *Leptospira* may rise as a public health concern, especially in relation to stray and kenneled dogs (DE PAULA DREER et al., 2013). Stray dog populations and dogs kept under shelter conditions are considered more susceptible to the infection in consequence of a higher environmental exposure to pathogenic *Leptospira* (SCANZIANI et al., 2002; JITTAPALAPONG et al., 2009) and high seroprevalence rates have been reported in sheltered dog populations worldwide (JITTAPALAPONG et al., 2009; DESVARS et al., 2012; CHEN et al., 2014), including stray and sheltered populations from Brazil (DE PAULA DREER et al., 2013; VIEGAS et al, 2001) .

São Paulo has become the second Brazilian state to enact a law banning the euthanasia of stray dogs (law n. 12.916, enacted in April 16, 2008). The law predicts that educational measures against relinquishment should be taken by local agencies and establishes adoption as the main alternative to control stray dog populations. However, few structural or financial investments were addressed towards local institutions to support proper removal and allocation of stray dogs. These circumstances led to the emergence of several overpopulated shelters, with high turnover of animals, often followed by remarkable stressful and unsanitary housing conditions. Moreover, admission or adoption protocols are frequently not implemented, predisposing the introduction and spread of leptospiral strains among housed dogs.

Poor sanitary conditions, infrastructure deficiencies and high rodent infestation are strongly related to higher prevalence of leptospiral infection in shelter facilities (SCANZIANI et al., 2002; JITTAPALAPONG et al., 2009; FUNG et al., 2014). Such conditions can represent increased chances of transmission among housed dogs as well as occupational risks to kennel workers and caretakers (AWOSANYA et al., 2013). Moreover, the rising trend for adoption in locations that banned euthanasia of stray dogs, associated with the increased risk of adopting infected dogs can hypothetically contribute to dog-to-human transmission by bringing pathogenic strains closer to adopters and their households (AWOSANYA et al., 2013; CRUZ-ROMERO; ROMERO-SALAS; AGUIRRE, 2013; GAY; SOUPÉ-GILBERT; GOARANT, 2014).

In order to promote evidence-based knowledge regarding asymptomatic urinary shedding of leptospires among stray and sheltered dog populations, the present study proposes the identification of chronically infected animals and the characterization of potentially pathogenic strains circulating among these populations. A quantitative PCR reaction targeting the *lipL32* gene was developed and validated to identify dogs presenting asymptomatic urinary shedding of leptospires, and qPCR-positive dogs were prospectively evaluated in order to confirm chronic infection as well as to recover viable leptospires for proper characterization. Prospective evaluations included blood and urine PCR testing, leptospiral culture from urine samples and detection of anti-*Leptospira* antibodies. Physical examination, serum biochemistry analysis and evaluation of hematologic parameters were also performed to exclude acute leptospiral infection. Leptospiruric dogs were reevaluated until presenting two negative consecutive urinary PCR results.

The study was conducted in three different dogs populations: (I) 92 dogs kept in a public shelter at the University of São Paulo campus, located in the west region of São Paulo city, Brazil; (II) seven stray dogs living inside the University of São Paulo campus; and (III) 24 dogs kept in a public shelter from the city of Mogi das Cruzes, located in the eastern region of São Paulo State, Brazil.

2 CONCLUSIONS

This study has successfully developed a highly sensitive and specific quantitative PCR assay in order to detect leptospiral DNA in urine samples taken from dogs. Identification of asymptomatic urinary shedding of pathogenic *Leptospira* was achieved in all populations studied, and prospective evaluation of leptospiruric dogs allowed the observation of persistent urinary shedding of leptospires, therefore evidencing asymptomatic chronic infection in at least three dogs. It also enabled the isolation of pathogenic *Leptospira* from two dogs characterized as chronic carriers.

Sequence analysis of the infecting strains found in leptospiruric animals revealed that the majority of dogs presented urinary shedding of pathogenic *Leptospira interrogans*. However, renal carriage of *Leptospira santarosai* was observed in dogs from the three populations studied. This is the first report of *L. santarosai* infection in dogs and suggests a possible and unexpected role of dogs in the chain of transmission of this particular pathogen in urban environments. Results also suggest a possible genetic distinction between *L. santarosai* maintained by dogs and other animal hosts, highlighting the plasticity of *L. santarosai* to infect such a variety of reservoir species. One of the dogs presenting *L. santarosai* infection was fully characterized as a chronic carrier, allowing recovery and proper identification of the infecting strain. This features as the first report of dogs persistently harbouring leptospiral species other than *L. interrogans* and might contribute to unravel the role of dogs in the transmission of this pathogenic *Leptospira* in urban scenarios.

This study has also identified that leptospiruric dogs can be inadvertently admitted and adopted in dog shelters, potentially increasing the risks of zoonotic transmission by bringing infected animals closer adopters and their households. Although the real contribution of dogs to the epidemiology of leptospirosis in urban centers is still not fully established, identification and proper management of chronically infected animals should be of public health concern.

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