BRUNO ALONSO MIOTTO

**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**

São Paulo

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Tese apresentada ao Programa de Pós-Graduação em Clínica Veterinária da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

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CERTIFICADO

Certificamos que o Projeto intitulado "Avaliação da leptospirúria e identificação de portadores de leptospiras patogênicas em cães mantidos em abrigos públicos ou particulares da região metropolitana de São Paulo", protocolado sob o nº2706/2012, utilizando 200 (duzentos) cães, sob a responsabilidade da Profa. Dra. Mitika Kuribayashi Hagiwara, foi aprovado "ad referendum" e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "Study of urinary shedding and identification of chronic carriers of pathogenic leptospiras in dogs kept in public or private animal shelters of metropolitan São Paulo area", protocol number 2706/2012, utilizing 200 (two hundred) dogs, under the responsibility Profa. Dra. Mitika Kuribayashi Hagiwara,, was approved "ad referendum" and agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo.

São Paulo, 20 de maio de 2015.

Denise Tabacchi Fantoni
Presidente

FOLHA DE AVALIAÇÃO

Autor: MIOTTO, Bruno Alonso

Título: **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 leptospirose e identificação de portadores de leptospiros patogênicos 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 leptospiros patogênicos e podem atuar como potenciais fontes de infecção da doença. A identificação de tais indivíduos e a caracterização de leptospiros 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 leptospiros patogênicos, 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 leptospiros 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 leptospiros 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 leptospiros 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 leptospiros. 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 leptospirose e identificação de portadores de leptospirose 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 leptospirosis 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*.

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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 asymptotically 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 epidemiological 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.

CHAPTER 1:
Development and validation of a modified qPCR assay targeting the
***lipL32* gene for the detection of pathogenic *Leptospira* in canine**
urine samples

2 DEVELOPMENT AND VALIDATION OF A MODIFIED QPCR ASSAY TARGETING THE LIPL32 GENE FOR THE DETECTION OF PATHOGENIC LEPTOSPIRA IN CANINE URINE SAMPLES

Ready for submission

2.1 INTRODUCTION

Leptospirosis is a re-emerging bacterial disease of global importance caused by pathogenic spirochaetes of the genus *Leptospira* (SCHULLER et al., 2015). Pathogenic *Leptospira* is currently classified into 10 distinct genomospecies and more than 260 serovars (BOURHY et al., 2014) that can affect a wide variety of mammalian species, including dogs and humans. Leptospiral transmission occurs through direct contact with contaminated biological material or indirectly by contacting contaminated environmental water resources (ANDRE-FONTAINE, 2006).

Canine leptospirosis is frequently reported worldwide (AZOCAR-AEDO, 2015; SCHULLER et al., 2015) and infected dogs manifest a broad range of clinical symptoms, varying from hepatic and renal failure, often accompanied by haemorrhagic and pulmonary disorders, to mild, self-limiting febrile illness (ANDRE-FONTAINE, 2006). Although susceptible to many serovars, dogs can act as maintenance hosts for different pathogenic *Leptospira* sp., notably *L. interrogans* sv. Canicola, contributing to the spread of leptospires into the environment without overt clinical signs of the infection (SCHULLER et al., 2015).

The diagnostic approach for canine leptospirosis is predominantly based on serological evidences of infection, usually performed by the microscopic agglutination test (MAT) (PICARDEAU et al., 2014). However, MAT presents several limitations to confirm leptospiral infection and MAT results should be interpreted with parsimony. MAT presents low sensitivity during the early stages of the disease, usually requiring multiple samples from acute and convalescent phases to confirm infection (LEVETT, 2001; MILLER et al., 2011) and serum titers found may reveal just a recent exposure to the pathogen instead of an actual active infection (MILLER et al., 2011). The identification of chronically infected individuals by

MAT can also be challenging; serum titers may not be necessarily associated with asymptomatic renal carriage of leptospires and the interpretation of serological data can be particularly confusing in dogs with recent or unknown immunization status (ANDRE-FONTAINE, 2006). Although culturing leptospires is still considered the eligible test to unmistakably confirm leptospiral infection, recovery of viable leptospires in culture media faces critical drawbacks, such as frequent contamination, fastidious growth of the pathogen and low diagnostic sensitivity (SCHULLER et al., 2015), undermining its use in clinical and epidemiological studies. PCR on the other hand has been successfully used to confirm leptospiral infection, enabling the early diagnosis of clinical leptospirosis (HARKIN; ROSHTO; SULLIVAN, 2003; HARKIN et al., 2003) as well as the identification of dogs presenting asymptomatic urinary shedding of leptospires (ROJAS et al., 2010; ZAKERI et al., 2010; GAY; SOUPÉ-GILBERT; GOARANT, 2014; SAMIR et al., 2015).

Due to its practical applicability, a great number of PCR assays targeting different protein-encoding genes have been developed for the diagnosis of human and animal leptospirosis (PICARDEAU et al., 2014). Different quantitative PCR protocols targeting the *lipL32* gene were previously described (STODDARD et al., 2009; ROJAS et al., 2010; VILLUMSEN et al., 2012; FINK et al., 2015); however, few studies regarded the validation of these assays to detect leptospires in canine specimens (ROJAS et al., 2010; FINK et al., 2015). LipL32 is an abundant outer membrane protein that occurs exclusively in pathogenic *Leptospira* and presents highly pairwise DNA sequence identity amongst pathogenic species (PINNE; HAAKE, 2013).

The current report describes the development and validation of a modified quantitative PCR assay targeting the *lipL32* gene for the identification of pathogenic *Leptospira* in canine urine samples.

2.2 MATERIAL AND METHODS

2.2.1 Samples and isolates

Urine samples from 35 dogs suspected of clinical leptospirosis attended at the University of São Paulo Veterinary Hospital Service (Hovet FMVZ-USP) between 2013 and 2015 were used to evaluate the diagnostic sensitivity and specificity of the qPCR test. Dogs included in this study were considered suspected for acute leptospirosis when presenting high BUN and creatinine levels (above 60 mg/dL and 1,4 g/dL, respectively) associated with two or more typical clinical manifestations of leptospirosis (such as hemorrhagic disorders, fever, jaundice, prostration or muscular weakness). Additional 116 urine samples from apparently healthy dogs kept in two distinct local public shelters collected during 2013 were also tested. The Ethical Committee of the College of Veterinary Medicine and Zootechny, University of São Paulo (protocols 2706/2012 and 2406140614) has approved all procedures involving animal manipulation in the current study.

Leptospiral strains used in this study were provided by the Laboratory of Bacterial Zoonosis, Department of Veterinary Preventive Medicine and Animal Health, College of Veterinary Medicine and Zootechny, University of São Paulo. The isolates were maintained in semisolid Fletcher media (BD Diagnostics, Sparks, MD) containing 1.5% agar (BD Diagnostics) at 28°C under aerobic conditions and collected at mid-log phase.

2.2.2 Sample processing and DNA extraction

Urine samples were collected via urinary catheterization or cystocentesis and centrifuged within 2 hours after collection (6,500xg for 25 min at 25°C); the pellets were resuspended in 2 ml of sterile Phosphate-buffered saline (PBS - pH 7.2) and all DNA extractions were processed at maximum 48 h after collection. Pure cultures of leptospires and non-spirochaetal pathogens used for testing the specificity of the test were subjected to two washing steps (6.500xg, 25 min at 25°C) using sterile PBS, as previously described (LEVETT, 2005; STODDARD et al., 2009). Genomic DNA from all samples included in the study was extracted using NucliSens® miniMAG™ (BioMérieux Inc., Durham, NC, USA), according to manufacturer's instructions, with slight modifications; 1 ml of resuspended solution was used in the initial lysis step and the final elution step was performed with 40µL.

In order to access the performance of the DNA extraction procedures, all clinical samples were submitted to a quantitative amplification targeting the Melanocortin 1 Receptor encoding-gene (MC1R), a nuclear gene that encodes for a seven-pass transmembrane G protein-coupled receptor protein involved in hair and fur coloring in mammals (EVANS et al., 2007).

2.2.3 Primers design and probe

The selection of primers and probe targeting the *lipL32* gene was based on a previously reported real-time PCR reaction described by Rojas *et al.* (2010). A total of 113 sequences encoding the LipL32 protein available at the National Center for Biotechnology Information Nucleotide Database (NCBI; <http://www.ncbi.nlm.nih.gov/>) were aligned using Mega 5.10 software (TAMURA et al., 2013). Selected sequences included all available *L. interrogans* sv. Canicola sequences, as well as sequences from *L. interrogans* serovars commonly attributed to canine leptospirosis (serovars Icterohaemorrhagiae, Pomona and Grippotyphosa) along with other representative serovars from *L. interrogans*, *L. kirschneri*, *L. borpetersenii*, *L. noguchii*, *L. weilii* and *L. santarosai* species (Supplementary material 1 - APPENDIX A). Two selected sequences (AY461924 and DQ286416) presented one nucleotide mismatch with the degenerated forward primer previously described (ROJAS et al., 2010); oligonucleotide structural analysis of the primer was performed using OligoAnalyser 3.1 (<http://www.idtdna.com/>) and also pointed out possible self-dimer and hairpin formation. In order to accommodate all available sequences tested and to overcome possible structural primer instability, a newly non-degenerated forward primer was designed (5'-TAAAGCCAGGACAAGCGCC-3'), generating a 138 bp amplicon when combined with the reverse primer previously described. The fluorescent probe sequence described by Rojas *et al.* (2010) was not modified, except for the use of MGB as a quencher at the 3'-end instead of NFQ.

2.2.4 qPCR assays

Optimal concentration of primers and probe targeting the *lipL32* gene were determined according to the manufacturer's recommendations (Applied Biosystems, Thermo Fisher Scientific Inc, Carlsbad, CA, USA), resulting in a final volume of 25 μ L per reaction using 600 μ M of each primer, 250 μ M of the probe, 1x TaqMan® Universal Master Mix II (Thermo Fisher, Scientific Inc, Carlsbad, CA, USA), DNase free-water and 5 μ L of extracted DNA. The amplification protocol consisted of two min at 50°C, 10 min at 95°C and 45 cycles of amplification (95°C for 15 s and 60°C for 60 s).

The MC1R assay was performed using primers and probe previously described by Kanthaswamy *et al.* (2012). The reaction consisted of 1x TaqMan® Universal Master Mix II (Thermo Fisher, Scientific Inc, Carlsbad, CA, USA), 900 μ M of each primer, 250 μ M of the probe, 2 μ L of sterile DNase free-water and 5 μ L of extracted DNA, resulting in a final volume reaction of 25 μ L. Cycling conditions consisted of an initial step of 50°C for two min, followed by 10 min at 95°C and 40 cycles of amplification (95°C for 15s and 60°C for 60s).

All qPCRs runs were performed in the same machine (Applied Biosystems® 7500 Real-Time PCR System, Thermo Fisher Scientific Inc, Carlsbad, CA, USA).

2.2.5 Analytical sensitivity of the *lipL32* assay

Analytical sensitivity of the *lipL32* assay was determined by the amplification of serial dilutions of genomic DNA extracted from a pure culture of *L. interrogans* serovar Canicola strain Hond Utrecht IV. Leptospire were quantified using a Petroff-Hausser counting chamber and DNA was extracted from a 2 ml sample containing approximately 7×10^8 leptospire/ml. Extracted DNA was quantified in duplicate using Qubit® 2 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) with a Quant-iT™ DNA Assay Kit (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA). The number of Genomic Equivalents (GE), assuming one genome copy per leptospire, was estimated based on a genomic size of 4.69Mb. For the preparation of standard-curves, extracted DNA was

standardized to an initial concentration of 2×10^6 GE/ μ L to perform serial 10-fold dilutions until reach 2×10^0 GE/ μ L. All standard-curve dilutions were tested in triplicate, and each run included a single negative control containing sterile, nuclease-free water. The lower limit of detection (LLOD) was determined as the concentration at which 95% or more of the replicates presented consistent amplification. C_t (cycle threshold) cutoff value was determined by calculating the average C_t values of LLOD replicates from different standard curve runs. Coefficients of variation (CV) and average C_t values were calculated to define inter and intra assay variations and estimate the assay reliability. The CV values were calculated using the standard deviation (SD) and mean C_t using the formula $CV = (SD \times 100\%) / \text{mean } C_t$.

2.2.6 Analytical specificity of the *lipL32* assay

The specificity of the new set of primers targeting the *lipL32* gene was initially confirmed by BLAST analysis (<http://blast.ncbi.nlm.nih.gov/>) followed by performing the assay using extracted DNA from several spirochaetes as template, including pathogenic strains, saprophytic strains and strains with intermediate pathogenicity (Table 1 – APPENDIX A). DNA extracted from pure cultures of other pathogens were also tested, including *Brucella canis*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella enterica* sv. *Typhi*, *Yersinia enterocolitica*, *Proteus mirabilis*, *Candida albicans*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Rickettsia rickettsii*, *Rickettsia bellii*, *Rickettsia parkeri*, *Ehrlichia canis*, *Leishmania infantum* and *Toxoplasma gondii*.

2.2.7 Diagnostic sensitivity and specificity of the *lipL32* assay

Quantitative amplification of DNA extracted from clinical samples were performed in triplicate and were considered positive when at least 2/3 replicates presented C_t values lower than the established cutoff value. For comparative analysis, the performance of the modified *lipL32* qPCR assay was compared to results obtained from a well-established conventional PCR using a *Leptospira* genus-specific set of primers targeting a 331 bp fragment of the 16S rRNA gene (MÉRIEN et al., 1992). Conventional PCR amplification was carried out as follows: one cycle at 94°C for 5 min, 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. Pure *L. interrogans* serovar Canicola (strain Hond Utrecht IV) genomic DNA was used as positive control and DNase-free water as negative control in all PCR runs. Amplified products were separated on a 2% agarose gel electrophoresis stained with SYBRSafe DNA Gel Stain (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) and analyzed by UV transillumination. The nucleotide sequences of the amplified fragments were sequenced using BigDye Terminator 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc, Carlsbad, CA, USA) on an ABI 7500 Genetic Analyzer (Thermo Fisher Scientific Inc, Carlsbad, CA, USA), according to manufacturer's recommendation. The obtained sequences were compared to publically available bacterial sequences using BLAST to determine species identification. Agreement between the assays was assessed using the Kappa test adopting a confidence interval of 95% along with the calculation of relative diagnostic sensitivity and specificity.

2.3 RESULTS

The modified *lipL32* assay was able to detect 2×10^5 GE/ μ L to 2×10^0 GE/ μ L of the extracted leptospiral DNA in a linear matter, with correlation efficiency (r^2) of 0.998 and efficiency of 98.96%. Analytic data also showed that the assay was able to consistently detect 10 copies per reaction (2×10^0 GE/ μ L): when analyzing 42 replicates containing 10 copies/reaction from eight different runs, 41 replicates presented amplification.

The reaction presented a cutoff C_t value of 37.5 arbitrary units (SD 0.324) and was determined based on the average C_t value of 17 replicates amplifying 10 copies per reaction

from five different standard curve runs. The standard curves were selected based on the adequate efficiency values (ranging from 92.74% to 95.30%) and high C_t values (>38) were not considered in the calculation to avoid unspecific amplifications. All selected runs were performed on separate days and the highest intrassay and interassay CV values considering all standard curve dilutions were 1.43 and 1.12, respectively (Supplementary Material 2 – APPENDIX A).

The assay was able to detect DNA from all pathogenic *Leptospira* sp. tested, while none of the intermediate or saprophytic *Leptospira* strains presented amplification (Table 1 – APPENDIX A). All strains tested were also subjected to the 16S rRNA amplification to confirm appropriate extraction procedure, and all samples yielded strong positive bands when analyzed in agarose gel (Table 1 - APPENDIX A). Moreover, the new set of primers used did not show unspecific hybridization in the *in silico* evaluation and no amplification was observed when DNA from other non-spirochetal pathogens were tested. The Relative diagnostic sensitivity, specificity and agreement between the *lipL32* and 16S PCR assays of all samples tested are presented in Table 2 (APPENDIX A).

Out of the 35 urine samples collected from dogs presenting with suspected acute leptospirosis, 11 (31.42%) showed positive results in the qPCR assay. All positive samples also tested positive for the 16S rRNA conventional PCR; DNA from all samples could be sequenced and were identified by BLAST as belonging to pathogenic *Leptospira* sp. However, five additional samples tested positive exclusively for the 16S rRNA amplification; three of them presented high sequence identity to non-leptospiral organisms (uncultured organisms and *Canis lupus familiaris*), and the other two PCR positive samples presented high sequence identity with pathogenic *Leptospira* sp, highlighting sensitivity differences between the assays. From the 116 samples collected from apparently healthy dogs, 11 (9.48%) tested positive in the qPCR assay; all positive qPCR samples also tested positive for the conventional PCR and were successfully identified as pathogenic *Leptospira* species by further sequencing analysis. Nevertheless, additional 15 urine samples presented positive results exclusively for the 16S rRNA PCR; three samples did not retrieved readable sequences, however sequence analysis from the other 12 samples revealed unspecific amplifications, with high sequence identity for *Collinsella intestinalis* (n=2), *Corynebacterium* sp. (n=2), *Pasteurellaceae bacterium* (n=1), uncultured organisms (n=6) and *Canis lupus familiaris* (n=1).

All *lipL32*-negative samples tested positive for the MC1R gene, thus confirming appropriate DNA extraction and amplification procedures.

2.4 DISCUSSION

The modified qPCR assay described in this study presented high analytical sensitivity, enabling consistent detection of low concentrations of leptospiral DNA extracted from canine urine specimens (at least 10 copies per reaction). These results are compatible to the sensitivity levels of previous studies using the *lipL32* gene as target for the molecular detection of leptospires in clinical samples (STODDARD et al., 2009; ROJAS et al., 2010; FINK et al., 2015). The reaction presented low analytical variation within or between runs, thus confirming high analytical reliability of the assay. The newly designed forward primer showed consensus areas with all sequences retrieved from GenBank, including the sequences from two leptospiral strains belonging to *L. santarosai* and *L. borgpetersenii* that presented nucleotide mismatches with the originally described forward primer (ROJAS et al., 2010). Although unusual, *L. santarosai* and *L. borgpetersenii* infection were recently reported in dogs (LLEWELLYN et al., 2016; SEE CHAPTERS TWO AND FOUR) and the development of highly sensitive assays capable to detect a more diverse range of pathogenic *Leptospira* sp. could be crucial for a wider application of molecular assays under clinical or epidemiological settings. The qPCR assay was able to detect DNA from all pathogenic *Leptospira* sp. tested, while none of intermediate and saprophytic strains presented amplification. Moreover, no *in silico* unspecific primer hybridization was detected and no amplification was observed when DNA from non-leptospiral pathogens were tested, thus confirming the high specificity of the assay.

All positive qPCR results obtained from clinical samples were confirmed by the conventional 16S PCR and further sequencing analysis. Despite the high overall agreement between the assays (kappa = 0.948), two samples tested positive exclusively for the 16S PCR; nucleotide sequences obtained from both of these samples presented high identity with *L. interrogans* species, thus confirming leptospiral infection. However, the performance of the qPCR assay was tested several months after the 16S PCR, and the discrepancy between the

results could be explained by a possible loss of DNA integrity during sample storage, as previously described (CARLSEN; JENSEN, 2010). Conventional PCR protocols are more susceptible to contamination during laboratorial procedures than real-time assays (MACKAY, 2004), and the differences observed can also be explained by a possible contamination while handling clinical material. When considering exclusively the *lipL32* assay, 24 of 35 dogs with suspected clinical leptospirosis did not present amplification of leptospiral DNA in urine samples. These results can be attributed to several causes, including (I) intermittent urinary shedding of leptospires via urine, (II) hiperacute manifestation of the disease, therefore preceding the pathogen migration to renal tubules, (III) clinical manifestations attributed to other causes not related to leptospiral infection and (IV) shedding of very low quantities of leptospires in the urine, restricting the detection of the pathogen by the assay.

Molecular evaluation of urine samples taken from asymptomatic dogs showed high agreement between real-time PCR and conventional PCR associated with nucleotide sequence analysis ($\kappa = 1$, data not shown), with high relative sensitivity and specificity values when comparing both assays (100% sensitive and specific, data not shown). Nonetheless, 15 samples tested positive exclusively for the conventional PCR assay, and sequencing analysis provided evidence for unspecific amplification in most of these samples ($n=12$). Despite several attempts, it was not possible to recover readable sequences from the three other samples. The 16S ribosomal RNA gene sequencing is the most usual approach for molecular characterization of spirochaetes and it is considered a well-established PCR-based tool to distinguish leptospiral species (MOREY et al., 2006). However, previous reports have shown considerable risks of amplifying DNA from certain commensal and environmental bacteria (FINK et al., 2015), undermining its use for diagnostic confirmation of leptospiral infection in clinical samples. Although the 16S PCR was shown to be remarkably unspecific in the present study, results also showed that the use of primers targeting the 16S gene can provide reliable results if associated with sequencing methods. All unspecific amplifications tested negative in the quantitative PCR, and the comparative analysis between the assays confirmed the high specificity of the newly developed qPCR assay.

The present study reported a reliable and highly sensitive quantitative assay targeting exclusively pathogenic *Leptospira* species. The validation of the assay using canine urine samples may contribute to a more precise diagnosis of dogs with suspected clinical leptospirosis. More importantly, it also enables the identification of asymptotically

infected dogs; renal carriage of pathogenic *Leptospira* is a well-described condition in the canine species (SCHULLER et al., 2015) and dogs presenting asymptomatic urinary shedding of leptospire may act as potential sources of bacterial transmission in urban and rural environments (GAY; SOUPÉ-GILBERT; GOARANT, 2014). The identification of such individuals remains crucial to prevent leptospiral spread into the environment.

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APPENDIX A

Table 1: Pathogenic and intermediate and nonpathogenic *Leptospira* strains used for sensitivity and specificity tests and results from qPCR and 16S PCR assays.

Species	Serogroup	Serovar	Strain	qPCR <i>lipL32</i>	PCR 16S
Pathogenic					
<i>L. interrogans</i>	Pomona	Pomona	pomona	+	+
	Canicola	Canicola	Hond Utrecht IV	+	+
	Icterohaemorrhagiae	Icterohaemorrhagiae	rga	+	+
	Icterohaemorrhagiae	Copenhageni	m20	+	+
	Sejroe	Hardjo (Hardjoprajitno)	hardjoprajitno	+	+
<i>L. kirschneri</i>	Autumnalis	Butembo	butembo	+	+
	Cynopteri	Cynopteri	3522c	+	+
	Pomona	Mozdok	m36/05	+	+
<i>L. santarosai</i>	Shermani	Shermani	1342k	+	+
	Grippothyphosa	Bananal	2a/cap	+	+
	Sejroe	Guaricura	guaicurus	+	+
<i>L. borgpetersenii</i>	Ballum	Castellonis	castellon 3	+	+
	Celledoni	Whitcombi	whitcomb	+	+
	Javanica	Javanica	veldrat batavia 46	+	+
	Mini	Mini	sari	+	+
<i>L. noguchii</i>	Panama	Panama	cz 214	+	+
	Panama		u73	+	+
Intermediate/Nonpathogenic					
<i>L. inadai</i>	Lyme		m34/99	-	+
<i>L. meyeri</i>			19cap	-	+
<i>L. biflexa</i>	Doberdo	Rufino	rpe	-	+
	Seramanga	Patoc	patoc	-	+
	Nazaré	Nazaré	nazaré	-	+
	Andamana	Bovedo	bovedo	-	+
		Buenos Aires	buenos aires	-	+
	Garcia	Garcia	garcia	-	+

Source: Miotto, B.A.; 2016.

Table 2: Calculation of Kappa test and comparative sensitivity and specificity between the *lipL32* qPCR, 16S PCR and 16S PCR associated with sequencing analysis used for diagnosis of leptospiral infection in symptomatic and asymptomatic dogs.

qPCR	16S PCR		Total	16S PCR + <i>Leptospira</i> sp. identification by sequencing		Total
	Negative	Positive		Negative	Positive	
Negative	108	20	128	127	2	129
Positive	0	23	23	0	22	22
Total	108	43	151	127	24	151
Sp=100% Se=53.4% Kappa= 0.593			Sp=100% Se=91.6% Kappa= 0.948			

Source: Miotto, B.A.; 2016.

Supplementary Material 1: Accession numbers from GenBank database used for primer and probe analysis

AB094433.2, AB094434.2, AB094435.2, AB094436.2, AB094437.2, AE010300.2, AF121192.1, AF181553.1, AF181554.1, AF181555.1, AF181556.1, AF245281.1, AJ580493.1, AR174015.1, AY423075.1, AY442332.1, AY461893.1, AY461894.1, AY461895.1, AY461896.1, AY461897.1, AY461898.1, AY461899.1, AY461906.1, AY461907.1, AY461908.1, AY461909.1, AY461910.1, AY461911.1, AY461912.1, AY461913.1, AY461914.1, AY461915.1, AY461916.1, AY461917.1, AY461918.1, AY461919.1, AY461920.1, AY461921.1, AY461922.1, AY461923.1, AY461924.1, AY461925.1, AY461926.1, AY461928.1, AY461929.1, AY461930.1, AY568679.1, AY568680.1, AY609321.1, AY609322.1, AY609323.1, AY609324.1, AY609325.1, AY609326.1, AY609327.1, AY609328.1, AY609329.1, AY609330.1, AY609331.1, AY609332.1, AY609333.1, AY776292.1, AY776293.1, CP000350.1, DQ149595.1, DQ092412.1, DQ286415.1, DQ286416.1, DQ286417.1, DQ286418.1, NC_004342, NC_008508, NC_008510, AF366366.1, AM937000.1, AY763509.1, EU871716.1, AY776294.1, U89708, AY223718.1, EU871720.1, EU871719.1, EU871718.1, EU871723.1, AE016823.1, JN831363, HM026175, AY609321, AY763509, DQ092412, AJ580493, AB094434, AY461909, AY423075, AF245281, AB094433, AY609324, AY461927, JN886738, AY609327, AF121192, EU871723

Supplementary Material 2: Inter and intratest parameters found between standard curve runs used to establish the cut-off values

Number of copies/reaction	Intra-test			Inter-test		
	Average Ct	SD	*VC (%)	Average Ct	SD	*VC (%)
1×10⁵	22.84 – 24.08	0.14 – 0.34	0.61 - 1.43	23.61	0.15	0.66
1×10⁴	27.00 – 27.00	0.01 - 0.16	0.15 - 0.59	27.00	0.00	0.00
1×10³	29.59 - 31.12	0,21 - 0.47	0.69 - 1.50	30.74	0.34	1.12
1×10²	33.80 - 34.69	0.08 - 0.37	0.23 - 1.09	34.26	0.24	0.71
1×10¹	37.20 - 37.72	0.10 - 0.29	0.27 - 0.78	37.50	0.07	0,19

* Variation Coefficiency. Source: Miotto, B.A.; 2016.

CHAPTER 2:

**Prospective study of canine leptospirosis at a shelter facility:
identification of chronically infected dogs and isolation of
different *Leptospira* species**

3 PROSPECTIVE STUDY OF CANINE LEPTOSPIROSIS AT A SHELTER FACILITY: IDENTIFICATION OF CHRONICALLY INFECTED DOGS AND ISOLATION OF DIFFERENT LEPTOSPIRA SPECIES

Ready for submission

3.1 INTRODUCTION

Leptospirosis is a re-emerging bacterial disease caused by pathogenic spirochaetes of the genus *Leptospira* (BOURHY et al., 2014). The disease is recognised as the most widespread zoonosis, and has emerged as a major public health issue in much of the developing world (COSTA et al., 2015). Human leptospirosis is frequently observed in poverty-stricken populations living in tropical regions (FELZEMBURGH et al., 2014), and it is considered one of the major neglected diseases in Latin America (HOTEZ et al., 2008). In Brazil, approximately 10,000 human cases are reported annually with overall case fatality of 10% among reported cases (FELZEMBURGH et al., 2014). However, diagnostic difficulties and unreported cases might overshadow the real burden of the disease, and the real incidence lean towards to be much higher (COSTA et al., 2015).

Pathogenic *Leptospira* are currently classified into more than 250 serovars and ten genomospecies (BOURHY et al., 2014). Virtually all mammalian species can be affected, and infection can cause a broad spectrum of clinical manifestations, ranging from severe and sometimes fatal outcomes to asymptomatic or mild self-limiting febrile illness (COSTA et al., 2015). Leptospire are maintained by different animal reservoirs and can be insidiously excreted in urine of chronically infected individuals (BARRAGAN et al., 2016), thus contributing to the spread of leptospire into the environment. The disease transmission is strongly driven by environmental factors, such as high pluviometric precipitation rates, flooding, natural disasters, uncontrolled urbanization expansion and poor sanitation and hygiene conditions (SCHNEIDER et al., 2015). The exposure to water and soil contaminated

by the urine of infected animals is the most common route of transmission to people and domestic animals (SCHNEIDER et al., 2015).

Rodents are considered the major source of human infection, a role likely attributed to its synanthropic behaviour and widespread distribution (COSTA et al., 2015). Nevertheless, recent “One Health” approaches have been used to circumvent crucial epidemiological aspects of leptospirosis, and several studies have pinpointed a significant role of different mammalian hosts in its zoonotic transmission (SCHNEIDER et al., 2015; BARRAGAN et al., 2016; GUERNIER et al., 2016; LAU et al., 2016).

Dogs are referred as natural reservoirs for pathogenic *L. interrogans* serovar Canicola (BRANGER et al., 2005) and asymptomatic urinary shedding of leptospires in canine populations has been widely reported (ROJAS et al., 2010). Nevertheless, dogs can also asymptotically shed a wide variety of *L. interrogans* serovars, such as Copenhageni and Icterohaemorrhagiae (SUEPAUL et al., 2009; GAY; SOUPÉ-GILBERT; GOARANT, 2014; SAMIR et al., 2015), Pomona (MACKINTOSH; BLACKMORE; MARSHALL, 1980; YASUDA et al., 1980; GAY; SOUPÉ-GILBERT; GOARANT, 2014), Tarassovi (MACKINTOSH; BLACKMORE; MARSHALL, 1980), Sejroe (SCANZIANI et al., 1995) and Hebdomadis (EVERARD et al., 1979), all potentially pathogenic to humans. Asymptomatic infection caused by *L. santarosai* (SEE CHAPTERS TWO AND FOUR), *L. kirschneri*, *L. borgpetersenii* and *L. wolffii* species were also reported (ZAKERI et al., 2010; DA CUNHA et al., 2016; HARKIN; HAYS, 2016; LLEWELLYN et al., 2016;), highlighting the unspecific nature of host-pathogen interactions.

Although the actual role of dogs in human leptospirosis is still debatable (MARTINS; PENNA; LILENBAUM, 2012), the close contact between dogs and human populations may offer favourable conditions for its zoonotic transmission (DAMBORG et al., 2015), a situation that might rise as a public health concern, especially in relation to stray and sheltered dogs.

Stray dog populations and dogs kept under shelter conditions are considered more susceptible to infection in consequence of a higher environmental exposure to pathogenic *Leptospira* (SCANZIANI et al., 2002; JITTAPALAPONG et al., 2009). In the past years it has been noticed a considerable increase in the number of dog shelters in Brazil, particularly in São Paulo state. This scenario comes as a result of a recent local law implementation (law n. 12.916, enacted in April 16, 2008), which has banned the euthanasia of stray dogs captured by animal control agencies, placing sterilization and adoption as the main legal strategies for dog population's control. These circumstances led to the emergence of several

overpopulated shelters, often followed by remarkable stressful and unsanitary housing conditions. Many of these shelters experience structural deficiencies and limited funding, whereas admission or adoption protocols are frequently not implemented, predisposing the introduction and spread of leptospiral strains among housed dogs (STENERODEN; HILL; SALMAN, 2011) and possibly increasing the chances for adoption of chronically infected dogs.

Proper management of chronic carriers is crucial to implement successful control strategies, however the identification of such individuals may be challenging. Serological data can be particularly confusing in dogs with unknown immunization status and serum titers may not be necessarily associated with renal carriage of leptospires, undermining its use for identification of leptospiruric dogs (ANDRE-FONTAINE, 2006). Alternatively, PCR has been successfully used to characterize asymptomatic urinary shedding of leptospires (ZAKERI et al., 2010). However, most reports are cross-sectional studies, restricting any considerations regarding the occasional, intermittent or persistent urinary shedding of the pathogen. The use of PCR as a screening test combined with further prospective evaluation of the infected animals may increase the chances of recovering leptospires for appropriate characterization; it can also provide clinical, laboratorial and serological data in order to characterize the dog's carrier status (SEE CHAPTER THREE). Characterization of local strains is critical for determining epidemiological aspects surrounding animal and human leptospirosis and can promote improved control and prevention strategies.

In order to evaluate leptospiral infection in dogs kept at a shelter with poor sanitary conditions, we designed a cross-sectional study to identify leptospiruric dogs, followed by a prospective study to properly characterize the chronic carrier state of the infected animals and to access the identity of the infecting strains.

3.2 MATERIAL AND METHODS

3.2.1 Study design and samples

Blood and urine samples were taken from 92 of 103 dogs kept in a public shelter at the University of São Paulo campus, located in São Paulo city, Brazil, between July of 2013 and January of 2014. Eleven dogs were extremely aggressive and were not included in the study.

Blood was collected from jugular or cephalic vein and drawn into BD Vacutainer tubes (BD Diagnostics) and Venosafe® tubes containing EDTA (Terumo). Whole-blood was used for qPCR testing; serum was recovered and used for biochemistry analysis and detection of anti-*Leptospira* sp. antibodies. Urine samples were taken by sterile urinary cystocentesis (males and females) or catheterism (males) and used for quantitative PCR testing.

Dogs presenting leptospiuria were prospectively evaluated to confirm persistence of infection. Reevaluations included blood and urine qPCR testing, leptospiral culture from urine samples and detection of serum anti-*Leptospira* antibodies. Clinical and laboratorial evaluations were also performed to access the clinical status of the infected dogs, and included physical examination, serum biochemistry analysis and evaluation of hematologic parameters. Follow-up of infected animals was interrupted only after presenting two negative consecutive qPCR results in urine samples. All chronically infected animals were treated after the study period using either a single subcutaneous dose of streptomycin 25mg/kg or an oral dose of doxycycline 4mg/kg for 14 days, according to previous recommendations (GREENE, 2011).

All procedures involving animals were approved by the Ethical Committee of the School of Veterinary Medicine of the University of São Paulo (protocol 2706/2012).

3.2.2 Study area

The shelter was designed to house 40 dogs appropriately. Additional 63 dogs were housed under improvised conditions into the administrative, maintenance, circulation and running areas. The facility had several inappropriate structural features, such as broken titers, water pump leakages and generalized build-up of rubble. There was no appropriate drainage of rain, organic waste or sewer collection and the vegetation coverage was overgrown,

shadowing the entire area. Many pens had no natural or artificial light and no proper ventilation, leading to permanent humidity. Pest control was carried out sporadically, however signs of high rodent infestation were present, such as bitten newspaper, droppings inside drawers and visualization of rodents at daylight.

The shelter had no organized admittance, quarantine procedure, medical care, sanitary, adoption, euthanasia or pain management protocols. All dogs admitted were sterilized before adoption and dogs were frequently immunized, but with no systematized protocol. The shelter had a relatively low turnover of animals, with approximately 4 dogs admitted and 4 dogs adopted per month.

3.2.3 Clinical and laboratorial evaluation

Clinical evaluation included inspection for jaundice, lymphadenopathy, dehydration and hyperthermia. The shelter staff was instructed to report any gastrointestinal, urogenital, cardio-respiratory, nervous and behavioral disorders during the study period.

Haematological analysis was performed in a ABX Micros ABC Vet (Horiba Medical) within two hours after sampling, and included white blood cell count (WBC) and platelet count (PLT). Differential WBC count was performed by optical microscopy of Rosenfeld-stained blood smears (Modified May-Grünwald). Serum biochemistry analysis was performed in a Labmax 240 device (Labtest Diagnostica) using Enzymatic Kinetic Method kit (Rx Series, Randox), following the manufacturer's specifications. The analysis included sera activity of alkaline phosphatase (AP) and alanine aminotransferase activity (AA). Total bilirubin (TB), total protein (TP), blood urea nitrogen (BUN) and creatinine (CR) serum concentrations were also determined. Reference intervals adopted for this study are presented in the APPENDIX B.

3.2.4 Microscopic agglutination test (MAT)

Antibody titration against *Leptospira* sp. was determined by MAT following previous

recommendation (World Organization for Animal Health, 2012) and included 22 serovars (Australis, Bratislava, Guaricura, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Hardjo-hardjoprajitno, Shermani, Tarassovi, Sentot) representing 18 serogroups.

Serum samples from 92 dogs were also subjected to MAT using leptospiral strains obtained (strains DU92 and DU114) in this study as antigens. Endpoint titers were determined by starting at an initial dilution of 1:100 and using two-fold dilutions until the last well showing 50% agglutination was recorded. The cutoff for a positive agglutination reaction was defined as a titer ≥ 100 .

3.2.5 Culturing of leptospires

For leptospires recovery, aliquots of urine samples were diluted in sterile physiological solution to a final concentration of 1:10 and 1:100 and 0.5 ml were inoculated into semi-solid Fletcher (Difco Laboratories, Franklin Lakes, NJ, USA) and liquid Ellinghausen, McCullough, Johnson, and Harris medium supplemented with 3% rabbit serum (EMJH - Difco Laboratories, Franklin Lakes, NJ, USA). All cultures were incubated at 28°C and examined weekly by dark-field microscopy for up to three months.

3.2.6 DNA extraction and PCR assays

Urine samples were centrifuged (10.000 xg, 25°C, 25 min) and pellets were resuspended in 2 ml sterile phosphate-buffered saline (PBS – pH 7.2) prior to storage at 4°C. DNA was extracted at maximum 48h after sample collection using Nuclisens MiniMag Kit[®] (BioMerieux, Inc., Durham, NC), with slight protocol modifications: 1 ml of the solution was used in the initial lysis step, and the final elution was performed using 40 µL. DNA from blood samples was extracted using QIAamp DNA Mini Kit[®] (Qiagen Inc., Valencia,

CA) following the manufacture's specifications. All extracted DNA were stored at -20°C until qPCR testing.

The detection of pathogenic *Leptospira* was performed using a quantitative real-time assay targeting the *lipL32* gene; primers (forward: 5'-TCGCTGAAATRGGWGTTCGT-3'; reverse: 5'-TAAAGCCAGGACAAGCGCC-3'), and probe (FAM-5'-AAAGCCAGGACAAGCGCCG-3'-MGB) were previously described (SEE CHAPTER ONE) and each reaction used 600 µM of each primer, 250 µM of the probe, 1x TaqMan® Universal Master Mix II (Thermo Fisher, Scientific Inc, Carlsbad, CA, USA), 5 µl of extracted DNA and DNase free-water for a final volume of 25 µL. The amplification protocol consisted of two min at 50°C, 10 min at 95°C and 45 cycles of amplification (95°C for 15 s and 60°C for 60 s). For absolute quantification, amplification of serial dilutions of genomic DNA extracted from *L. interrogans* serovar Canicola strain Hond Utrecht IV were performed along with clinical samples. Cultured leptospires were quantified using a Petroff-Hausser counting chamber and extracted DNA was quantified in duplicate using Qubit® 2 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) to determine the number of Genomic Equivalents (GE). For the preparation of standard-curves, extracted DNA was standardized to an initial concentration of 2×10^6 GE/µL to perform serial 10-fold dilutions until reach 2×10^0 GE/µL (10 copies/reaction). DNA from clinical specimens and standard curves were tested in triplicate. Each run included a single negative control containing PCR water and results were considered positive if cycle threshold (Ct) values were recorded in at least 2/3 replicates.

In order to access the quality of DNA extraction, all samples were submitted to a quantitative assay targeting the canine Melanocortin 1 Receptor encoding-gene (MC1R), as described elsewhere (KANTHASWAMY et al., 2012). Clinical specimens and positive controls using DNA extracted from pure cultures of canine fibroblast cells were tested in duplicate. Each run included a single negative control containing PCR water.

All qPCRs runs were performed using the same equipment (Applied Biosystems® 7500 Real-Time PCR System, Thermo Fisher Scientific Inc, Carlsbad, CA, USA).

To confirm qPCR results, all positive samples were submitted to a partial 16S rRNA gene amplification targeting a 331bp fragment (MÉRIEN et al., 1992; AHMED et al., 2006) and thereafter sequenced to confirm leptospiral sequence identity. PCR amplification was carried out with one cycle at 94°C for 5min, 40 cycles at 94°C for 30s, 60°C for 30s, 72°C for 30s and a final extension at 72°C for 5min. *L. interrogans* sv. Canicola genomic DNA

was used as positive control and DNAase-free water as negative control in all PCR runs.

3.2.7 Characterization of the isolated *Leptospira* strains

3.2.7.1 Molecular characterization

Species identification of the isolated strains was performed by Multilocus Sequence Typing (MLST) using seven distinct loci (*pntA*, *sucA*, *mreA*, *glmU*, *caiB*, *tpiA*, *pfkB*), as previously described (BOONSILP et al., 2011). Sequence types (STs) were determined from the resulting allelic profiles and compared to an established Internet database to obtain serovar identification (<http://pubmlst.org/leptospira>).

The 16S rRNA amplicons were sequenced and sequences were compared to reference strains deposited on GenBank using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3.2.7.2 Serological Characterization

The isolated strains were serogrouped using polyclonal antibodies according to previous recommendation (DIKKEN; KMETY, 1978). A panel of 34 specific antisera representing 28 serogroups was used (Andamana, Australis, Autumnalis, Ballum, Bataviae, Canicola, Caledoni, Codice, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Holland, Icterohaemorrhagiae, Javanica, Lousiana, Lyme, Manhao, Mini, Panama, Pomona, Pyrogenes, Ranarum, Sarmin, Sejroe, Seramanga, Shermani, Tarassovi). Serogroup identification was confirmed by the highest titrations against specific representative serovars included in the panel.

3.2.7.3 Virulence characterization

Pure cultures of each isolated strain were counted in a Petroff-Hausser chamber and 0.5 ml containing 10^8 leptospires was intraperitoneally inoculated in thirty-day-old male hamsters and guinea pigs to determine if the isolates would produce infection (JORGE et al., 2015). The animals were daily monitored for signs of acute leptospiral infection, including prostration, isolation, ruffed hair coat, jaundice, external haemorrhage and dehydration. Animals were euthanized after showing two or more clinical signs or after 21 days post-infection. Kidneys were aseptically removed, macerated, resuspended and inoculated in liquid EMJH medium for reisolation. Kidney tissues from animals presenting no signs of infection were also used for PCR testing and serum samples were used to investigate the presence of anti-*Leptospira* antibodies by MAT.

The DU114 strain was inoculated in two hamsters; the DU92 strain was inoculated in one hamster, followed by seven further *in vivo* passages; leptospires recovered from the last *in vivo* passage were also inoculated in four guinea pigs to evaluate the strain pathogenicity in a different animal model.

3.2.8 Sequencing PCR products

The MLST and 16S rRNA amplicons were separated on a 2% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, EUA) to further observation using UV transillumination. DNA fragments were purified using Wizard® SV gel and PCR Clean-up System (Promega Corporation, Madison, EUA) and the DNA sequencing was carried out on an ABI 7500 Genetic Analyser (Applied Biosystems Foster City, CA, USA) using Big Dye Terminator 3.1 Cycle Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA), according to manufacturer's instructions.

3.2.9 Statistical and phylogenetic analysis

MAT and quantitative qPCR results were analyzed (Pearson's chi-square test for qualitative data and Mann-Whitney test for quantitative data) using Sigma Stat for Windows Version 3.0 (SPSS, Inc.). The $p < 0.05$ was considered statistically significant.

For 16S rRNA phylogenetic analysis, obtained consensus sequences were aligned with GenBank reference sequences and phylogenetic trees were constructed using Mega 5.10 software (TAMURA et al., 2011) and Maximum-Likelihood method with Nearest Neighbour Interchanges; 1000 bootstrap replicates were used for branch support statistical inference. For MLST analysis, the concatenated loci were compared to *Leptospira* sequence types (STs) available on the PubMLST database using Maximum-Likelihood method by Bionumerics 7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium).

3.3 RESULTS

3.3.1 Cross-sectional evaluation

Serum antibodies against *Leptospira* sp. were detected in 47/92 dogs (51%), with titers ranging from 100 to 12,800. Most dogs had antibodies against serogroup Icterohaemorrhagiae (n=39), followed by Autumnalis (n=27) and Pomona (n=21). Less common serogroups were Canicola, Pyrogenes and Shermani (n=1 for each serogroup). From 92 dogs, 36 had been immunized against leptospirosis less than six months before samples were taken and 33 dogs had been immunized more than six months before sample collection. Immunization status from 23 dogs could not be determined: 11 dogs were recently admitted at the facility and 12 dogs didn't have immunization records. Table 1 (APPENDIX B) shows the highest titers found in MAT positive dogs according to their immunization records. There was a statistical difference in MAT results between the immunization categories ($p = 0.0009$): presence of antibodies was more frequent in recently vaccinated dogs when compared to dogs immunized more than six months before sample collection and dogs with unknown immunization status ($p = 0.0081$ e $p = 0.0021$, respectively). There was no statistical difference between dogs with no immunization records and dogs vaccinated more than six months before sample collection ($p > 0.05$).

DNA from pathogenic *Leptospira* sp. was amplified in 10/92 dogs (10.87%). Median volumes of qPCR-negative urine samples (9.8 ml) were not significantly higher when compared to median volumes of PCR-positive urine samples (10.5 ml), and the number of leptospires found in qPCR-positive dogs ranged from 23 to 682,545 leptospires/ml (Table 2 – APPENDIX B). No amplification of leptospiral DNA in blood samples or clinical abnormalities related to canine leptospirosis was observed among dogs included in this study, with the exception of dog C, which presented slight pale mucous membranes during examination. All negative urine samples and all blood samples tested positive for the MC1R assay and standard curves tested positive in all qPCR runs, indicating adequate DNA extraction and amplification procedures. The number of leptospires/ml urine, MAT results, laboratorial findings and relevant information regarding registry and immunization status of qPCR-positive dogs are presented in Table 2 (APPENDIX B).

The phylogenetic analysis of 16S rRNA from pathogenic *Leptospira* detected in all leptospiruric dogs enabled the identification of two species: *L. interrogans* and *L. santarosai* (Figure 1 - APPENDIX B). Nine of the recovered sequences were identified as *L. interrogans* (dogs A, C, D, E, F, G, H, I and J), presenting high similarity (> 99%) with *L. interrogans* serovar Canicola strain Hond Utrecht IV (AY996798) and *L. interrogans* serovar Pomona strain Pomona (AY996800). The remaining sequence (dog B) presented > 98% similarity with *L. santarosai* serovar Shermani strain LT821 (AY631883) and *L. santarosai* serovar Georgia strain LT117 (AY996805). The recovered sequences were submitted to GenBank under accession numbers KU682051 and KX891325 - KX891333.

3.3.2 Prospective evaluation

Nine from 10 leptospiruric dogs were regularly reevaluated, with mean interval of 10 days between evaluations (SD 4,57); one dog (dog A) was adopted after the first evaluation, sidelining prospective investigation. qPCR results from urine samples of all reevaluated dogs are presented in Table 3 (APPENDIX B). Seven leptospiruric dogs presented inconsistent urinary shedding of leptospires throughout the evaluations (Table 3 - APPENDIX B). No seroconversion, positive blood qPCR results or clinical/laboratorial abnormalities were

observed during the reevaluations of these dogs and It was not possible to isolate leptospires in culture media from these animals, even from those with qPCR-positive results.

However, two dogs (dogs B and C) presented persistent urinary shedding of leptospires, with positive qPCR results in several occasions (Table 3 - APPENDIX B). Dog B (male, adult, unvaccinated) was evaluated in 14 occasions throughout a 18 week-period, with mean interval of 8,5 days between evaluations (ranging from 7 to 14 days). Urinary qPCR tested positive in all samples and leptospires were recovered from two different occasions. Figure 2 (APPENDIX B) shows the number of leptospires/ml detected in each evaluation along with results from isolation attempts. After the 14th evaluation, the dog was properly treated with streptomycin (Estreptomax[®], Ourofino, Cravinhos, SP, Brazil) and no leptospiral DNA was detected in urine samples collected seven and 14 days post-treatment. No antibody titers against *Leptospira* were detected during the evaluations. The dog did not present any relevant clinical/laboratorial abnormalities and no amplification of leptospiral DNA was observed in blood samples throughout the evaluation period.

Dog C (male, adult, unknown immunization status) was evaluated in 10 occasions throughout a 12 week-period, with mean interval of 7.64 days between evaluations (ranging from 2 to 14 days). The dog was captured inside the University campus and had samples taken immediately after admittance. Dog C presented slightly pale mucous membranes during the first evaluation and was immunized against leptospirosis during the admission procedure. Leptospiral DNA was detected in urine samples from all evaluations. Dog C presented significantly more leptospires in urine samples than dog B throughout the evaluations ($p = 0.002$) and leptospires were recovered from five different occasions (Figure 2 – APPENDIX B).

Laboratorial evaluation of dog C revealed blood smear visualization of *Anaplasma* sp, low platelet count, low hematocrit and high BUN and creatinine levels in more than one occasion (Table 4 – APPENDIX B). After the 10th evaluation, the dog was properly treated using doxycycline (Doxitrat[®], Agener, Embu, SP, Brazil). No leptospiral DNA was detected in urine samples taken seven and 14 days after antibiotic intervention.

All urine and blood samples taken during revaluations of dogs B and C that presented no amplification for pathogenic *Leptospira* tested positive for the MC1R gene.

3.3.3 Characterization of the isolated strains

One isolate recovered from each dog was selected for further molecular and serological characterization. The isolated strain recovered from dog B was named DU92 and the isolated strain recovered from dog C was named DU114.

MLST analysis of DU114 strain revealed a ST37 profile, which characterizes *L. interrogans* serovar Canicola strains according to previously described protocol (BOONSILP et al., 2011); Figure 3 (APPENDIX B) corroborates the clustering of DU114 isolate with *L. interrogans* serovar Canicola STs. Serogrouping also revealed strong and specific titration against serogroup Canicola (12,800 for serovar Canicola, 3,200 for Icterohaemorrhagiae, and 800 to serovars Castellonis, Mini and Pyrogenes) and the strain was virulent in hamster model. Two hamsters inoculated with the isolated strain developed acute lethal infection within five days post-inoculation, and macroscopic alterations included epistaxis, generalized petechial stains, pulmonary/liver congestion and pulmonary haemorrhage. Leptospire were successfully recovered from kidney and liver tissues from both hamsters after the euthanasia procedure.

Characterization of the strain DU92 was previously performed by our group (SEE CHAPTER THREE), and revealed a strong and specific reaction against Sejroe serogroup. The MLST analysis revealed a new sequence type (ST218), characterizing the strain as *L. santarosai* (Figure 3 - APPENDIX B). The hamster inoculated with the DU92 strain did not present any clinical signs of acute leptospirosis. However, leptospire were recovered in culture media from kidney tissues after 21 days post-inoculation. Seven *in vivo* passages were performed using the DU92 without producing clinical signs of infection. Similarly, none of the four guinea pigs inoculated with the strain presented clinical signs of leptospirosis. It was not possible to recover leptospire from kidney samples 21 days after experimental infection, and none of the guinea pig kidney samples presented amplification for the 16S rRNA PCR. However, MAT titers exclusively against serogroup Sejroe were detected in 5/8 animals (2/4 hamsters and 3/4 guinea pigs), ranging from 100 to 400 against the serovars Wolffii and Hardjo.

3.3.4 MAT results using the local isolates

Serum samples from all dogs included in the study were retested by MAT using the isolates DU92 and DU114 as antigens. Out of 92 samples previously characterized using the standard MAT panel, none reacted with the local isolates.

3.4 DISCUSSION

High overall antibody prevalence (47/92) was observed in the cross-sectional evaluation of the population studied, corroborating to seroprevalence rates observed in sheltered dogs from different locations (JITTAPALAPONG et al., 2009; DESVARIS et al., 2012; CHEN et al., 2014). However, MAT results should be interpreted with parsimony; the majority of seroreactive dogs had been recently immunized (at least six months before sample collection), with most dogs reacting against *Icterohaemorrhagiae*, *Autumnalis* and *Pomona*, serovars which are frequently used in vaccine compositions or commonly associated with post-vaccinal cross reactions (GREENE, 2011; MARTIN et al., 2014). Moreover, the highest titers ($\geq 1,600$) were present exclusively in serum from recently vaccinated dogs ($n=6$), and although post-vaccinal titers are usually low and only persists for few months (MILLER et al., 2011), high titration during the early post-vaccination period has been reported (BARR et al., 2005). Surprisingly, only one dog had serum titers against *Canicola* serogroup, despite the immunization status. This apparently contradicts the high frequency of seroreactive dogs with titers against *Canicola* found in serological surveys of stray and sheltered dogs (CRUZ-ROMERO; ROMERO-SALAS; AGUIRRE, 2013; CHEN et al., 2014); however it still can be supported by previous studies showing that dogs immunized with vaccines containing *Canicola* strains can present low or no titers against this serovar (HARTMAN; VAN HOUTEN; FRIK, 1984; SCANZIANI et al., 2002).

The proportion of leptospirosis found in this study (10/92) was similar to urinary shedding rates previously reported (HARKIN et al., 2003; ROJAS et al., 2010; GAY; SOUPÉ-GILBERT; GOARANT, 2014). However, detection of leptospiral DNA does not predict bacterial

viability, and the intermittent pattern of urinary shedding observed in reservoir hosts can misrepresent the real prevalence of leptospiuria in the studied population. In this study, only five from 10 leptospiruric dogs presented concomitant MAT titers, from which two dogs had been recently vaccinated, highlighting no association between the presence of antibodies against *Leptospira* and urinary shedding of leptospires. These results corroborates previous reports showing that MAT is not an suitable technique for the identification of asymptomatic leptospiral infection (GREENE, 2011; HARKIN et al., 2003).

Sequence analysis of the 16S rRNA gene fragment confirmed leptospiral infection in all qPCR-positive dogs, also revealing infection caused by two different leptospiral species: *L. interrogans* (9/10 dogs) and *L. santarosai* (1/10 dogs). No remarkable clinical/laboratorial abnormalities were observed, and most dogs were considered clinically healthy (with the exception of dog B, which is further discussed).

Prospective evaluation revealed inconsistent leptospiuria in most dogs, possibly reflecting transient infection or intermittent urinary shedding of leptospires. It was not possible to characterize the infecting strains from intermittent shedders at serovar or serogroup level. However, two dogs presented persistent urinary shedding of leptospires (dog B and C), enabling further investigation regarding the infecting strains.

Dog B was successfully characterized as a chronic carrier, presenting no *lipL32* amplification from blood samples, no clinical signs of infection and no titration against *Leptospira* sp. throughout the evaluation period. Prolonged urinary shedding allowed recovery of leptospires in more than one occasion, and the strain DU92 was characterized as *L. santarosai* serogroup Sejroe. Serological and molecular characterization of isolate DU92 was previously described by our group (SEE CHAPTER THREE), featuring as the first reported *L. santarosai* strain ever isolated from a dog.

Virulence of DU92 strain could not be demonstrated in animal model, with both hamsters and guinea pigs failing to demonstrate signs of leptospirosis. Even so, serum titers against Sejroe serogroup were detected, evidencing that the strain can actually produce a specific immune response after experimental inoculation. Symptomatic and asymptomatic infection caused by serogroup Sejroe had been previously reported in dogs (SCANZIANI et al., 1995; RÜHL-FEHLERT et al., 2000), evidencing horizontal transmission among dogs kept under shelter conditions (SCANZIANI et al., 1995). In the present study, only one dog presented *L. santarosai* infection, with no evidence for horizontal transmission of this particular strain

among housed dogs. No serum titers against Sejroe serogroup were detected in any dog kept at the shelter, even when retesting MAT using the DU92 strain as antigen. Nonetheless, our group has recently identified stray and sheltered dogs from different locations also presenting asymptomatic urinary shedding of *L. santarosai* (SEE CHAPTER FOUR), with all available *L. santarosai* sequences recovered from dogs showing high nucleotide identity, raising the hypothesis that dogs can actually play a role in the transmission chain of this pathogen. *L. santarosai* has been increasingly identified as the causative agent of severe cases of human leptospirosis (BOURHY et al., 2013; VALVERDE et al., 2013; NAOTUNNA; AGAMPODI; AGAMPODI, 2016), and canine infection caused by this leptospiral species requires further investigation.

Dog C presented prolonged urinary shedding of leptospires confirmed by qPCR despite immunization, allowing recovery of leptospires in several occasions (strain DU114). The strain was characterized as *L. interrogans* sg. Canicola, which was demonstrated to be pathogenic in hamster model. Dogs are considered natural reservoir for serovar Canicola, and human cases of leptospirosis attributed to this serovar have also been described (TREVEJO et al., 1998; WANG et al., 2015). The usage of DU114 strain as antigen to retest serum samples from the 92 dogs included in the study has failed to increase seropositivity against serovar Canicola. It has been suggested that the inclusion of local strains as antigens in MAT panels may increase the serodiagnostic sensitivity of leptospirosis (PINTO et al., 2015), however contradictory results were also reported (MURRAY et al., 2011). These results suggest high host adaptation of Canicola strains to infect dogs without overt an immune response.

Dog C presented moderate increase in serum BUN/creatinin levels and haematological abnormalities combined with urinary shedding of leptospires during the first evaluation. Acute leptospirosis was suspected, however BUN/creatinin levels decreased rapidly during the following evaluations and no leptospiral DNA could be amplified from blood samples taken from any evaluation performed. Moreover, no remarkable clinical abnormalities were observed, and serum *Leptospira* titers found during the reevaluations were probably attributed to immunization procedure performed immediately after the dog's admission. Nevertheless, the persistence of relatively low platelet and haematocrit values and the severe low platelet count observed during the 9th evaluation led our group to investigate possible causes for haematological disturbances. Retrospective visualization of

blood smears revealed *Anaplasma sp.* morulae infecting platelets in blood samples taken from different occasions, and SNAP 4Dx® (Idexx, Westbrook, USA) confirmed *Anaplasma platys* infection. These findings suggest that hematological abnormalities presented by dog C were possibly caused by *A. platys* infection. The dog was promptly treated with doxycycline after confirmation of co-infection, and two urinary qPCR for pathogenic *Leptospira* tested negative after the drug intervention. The use of Doxycycline is recommended against several bacterial infections in dogs, including *Anaplasma* and *Leptospira* infections (GREENE, 2011).

Although consecutive positive qPCR results evidenced successful renal colonization by both isolated strains, significantly different mean quantities of leptospiral DNA were identified in urine samples from each dog. Dog C presented significantly higher numbers of leptospires in urine than Dog B, which was infected by a supposedly non-adapted serovar to persistently infect dogs. These results may reflect different host-pathogen interactions, but no profile or pattern of bacterial shedding can be further defined based exclusively on these data. Despite those differences, dogs with prolonged urinary shedding of pathogenic *Leptospira sp.* can promote continuous environmental contamination.

Historically, São Paulo has become the second Brazilian state to enact a law banning the euthanasia of stray dogs. However, the law does not predict structural or financial investments addressed to local institutions in order to support proper allocation of stray dogs. As a consequence, high animal density has become a systematic issue for many local shelters. Besides the low overall turnover of animals at the USP shelter, it became obvious that adoption could not balance the admission flow in a long-term basis, consequently leading to permanent overpopulated stalls. Most unsanitary conditions and animal welfare issues observed were caused or aggravated by inadequate allocation of dogs. Such conditions are associated with higher prevalence of leptospiral infection among sheltered dogs can represent serious occupational risks to kennel workers (SCANZIANI et al., 2002; AWOSANYA et al., 2013; FUNG et al., 2014).

From ten dogs identified as urinary shedders, two were recently admitted at the facility, including dog C. It was also noticed that one dog (dog A) was adopted immediately after presenting high quantities of leptospires in urine, evidencing that leptospiruric dogs can be inadvertently admitted and adopted in dog shelters. Adoption of infected animals can potentially increase the risks of dog-to-human transmission by bringing pathogenic strains closer to care givers, adopters and their households and should be of public health concern

(AWOSANYA et al., 2013; CRUZ-ROMERO; ROMERO-SALAS; AGUIRRE, 2013; GAY; SOUPÉ-GILBERT; GOARANT, 2014).

Besides vaccination can potentially decrease the risk of infection (KLAASEN et al., 2013), its use as a single strategy to prevent the leptospiral infection can be only partially effective in shelter environments, as our results have shown. Simultaneous control strategies should be implemented to prevent canine leptospirosis, such as highly standardized hygiene protocols, rodent infestation control and proper management of asymptotically infected dogs. Nevertheless, identification of leptospiruria requires specific laboratorial techniques (e.g. PCR, culture, dark field microscopy) that are not often available in a shelter daily basis. Nevertheless, asymptomatic infection can be effectively treated with appropriate antibiotics (GREENE, 2011), and both drug protocols used in this study were able to interrupt urinary shedding of leptospires. Despite being effective and practical in a shelter routine, the use of streptomycin is not recommended in several locations for its potential nephrotoxicity (JUVET et al., 2011). Doxycycline is currently considered the elective drug to prevent leptospiral shedding (GREENE, 2011), however, it requires a 14-day oral treatment at minimum, limiting its use in quarantine and adoption protocols at shelters with high turnover of animals. Results reported here highlights that efforts should be addressed to evaluate alternative drug protocols suitable for a shelter routine, allowing secure, practical and efficient use of antibiotics in order to prevent adoption and admission of asymptotically infected animals.

This work has successfully identified asymptomatic urinary shedding of pathogenic *Leptospira* in sheltered dogs. Prospective evaluation of the infected individuals allowed proper characterization of the chronic carrier state of two dogs, enabling also the isolation and the identification of the infecting strains. Results reported here can promote better understanding of epidemiological aspects of leptospirosis in urban environments and the role of dogs in the epidemiology of the disease. It also addresses that the implementation of prophylactic control can potentially contribute to overcome the diagnostic difficulties involving the identification of dogs presenting asymptomatic urinary shedding of leptospires, and should be considered by shelter administrators and public health agencies.

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APPENDIX B

Table 1: MAT results and maximum serum antibody titers against *Leptospira* sp. grouped according to immunization status found in 92 dogs housed at the PUSP-C shelter

Interval between immunization and sample collection	MAT Negative	MAT Positive (≥100)	Maximum MAT titration found in MAT positive dogs					TOTAL
			100	200	400	800	≥1,600	
<6 months	9	27	4	9	7	1	6	36
>6 months	20	13	3	7	3	0	0	33
Unknown	16	7	0	3	2	2	0	23
TOTAL			7	19	12	3	6	92

Source: Miotto, B.A.; 2016.

Table 2: relevant information regarding qPCR and MAT results, laboratorial findings and registry data from all dogs presenting urinary shedding of leptospires

Dog ID	Leptospire/ml urine	Interval between check-in and sample collection	Interval between immunization and sample collection	Major laboratory and clinical findings	MAT						
					PO	BU	CAS	IC	PY	BRA	AUT
A	682,545	2 months	1 month	Unremarkable	800	-	-	800	800	100	100
B	44	> 6 months	Up to 6 months	Unremarkable	-	-	-	-	-	-	-
C	137,183	1 hour	Unknown	See table 4	-	-	-	-	200	-	-
D	30	>6 months	4 months	Unremarkable	-	-	-	-	-	-	100
E	118	>6 months	Up to 6 months	Unremarkable	-	-	-	-	-	-	-
F	23	>6 months	Up to 6 months	Unremarkable	-	-	-	-	-	-	-
G	28	>6 months	Up to 6 months	Unremarkable	-	-	100	100	200	-	100
H	30	>6 months	Up to 6 months	Unremarkable	-	-	-	-	-	-	-
I	273	>6 months	1 month	Unremarkable	400	400	400	-	-	-	-
J	2,046	1 hour	Unknown	Unremarkable	-	-	-	-	-	-	-

PO: sv. Pomona; BU: sv. Butembo; CAS: sv. Castellonis; IC: sv. Icterohaemorrhagiae; PY: Pyrogenes; BRA: sv. Bratislava; AUT: sv. Autumnalis. Source: Miotto, B.A.; 2016.

Table 3: Prospective qPCR results from all leptospiruric dogs identified during the cross-sectional study.

Dog ID	Urine qPCR results/evaluation													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+				
D	+	-	-											
E	+	-	+	-	-									
F	+	-	-											
G	+	-	-											
H	+	+	-	-										
I	+	-	-											
J	+	-	-											

Source: Miotto, B.A.; 2016.

Table 4: Relevant laboratorial findings and MAT results found in samples taken during evaluations of dog C

Evaluation	Laboratorial findings				MAT								
	BUN (mg/dL)	Creat. (mg/dL)	PLT (10 ³ /mm ³)	Blood smear findings	PY	BRA	CAS	IC	PO	GRI	CA	BU	AUT
1	185,7	2,28	180	<i>A. platys</i> (+++), reactive linf. (+++), rective Mn. (+)	200	-	-	-	-	-	-	-	-
2	122,5	1,38	134	reactive linf. (+++), rective Mn. (++)	100	-	-	-	-	-	-	-	-
3	93	1,38	238	reactive linf. (+++), rective Mn. (++)	800	400	-	-	-	-	-	-	-
4	90	1,43	173	reactive linf. (++) , rective Mn. (+)	200	400	-	-	-	-	-	-	-
5	WRR	WRR	190	<i>A. platys</i> (+++), reactive linf. (+), rective Mn. (+)	200	200	-	100	100	-	-	100	-
6	WRR	WRR	140	reactive linf. (+++), rective Mn. (+)	-	200	-	-	200	-	-	-	-
7	WRR	WRR	152	rective Mn. (+)	-	-	200	100	-	100	400	-	100
8	WRR	WRR	100		-	-	200	100	-	100	400	-	100
9	WRR	WRR	36		-	-	400	200	-	-	200	-	200
10	WRR	WRR	224		-	-	200	-	-	-	400	-	100

WRR: Within reference range; PY: Pyrogenes sv.; BRA: Bratislava sv.; CAS: Castelonis sv.; IC: Icterohaemorrhagiae sv.; PO: Pomona sv.; GRI: Grippothyphosa sv.; CA: Canicola sv.; BU: Butembo sv.; AUT: Autumnalis sv. Source: Miotto, B.A.; 2016.

Figure 1: *Leptospira* species confirmation based on 16S rRNA phylogenetic analysis using the Maximum-Likelihood method with Nearest Neighbour Interchanges. The bootstrap values presented at corresponding branches were evaluated using 1000 replicates

Source: Miotto, B.A.; 2016.

Figure 2: Number of leptospires per ml urine and isolation results from samples taken from dog b and dog C throughout the evaluations

Source: Miotto, B.A.; 2016.

Figure 3: Maximum-likelihood tree based on the concatenated sequences of the seven loci for the 229 available *Leptospira* STs.

Source: Miotto, B.A.; 2016.

Reference ranges adopted for this study

Parameters	Units	Reference ranges	
ERITROGRAM		Minimum	Maximum
Erythrocytes	$\times 10^6/\mu\text{L}$	5,0	8,0
Haemoglobin	g/dL	12,0	18,0
Haematocrit	%	37	57
*MCV	fL	60,0	77,0
** MCH	pg	22,0	27,0
*** MCHC	%	31,0	36,0
LEUCOGRAM			
Total Leucocytes	$/\mu\text{L}$	6.000	15.000
Neutrophils	$/\mu\text{L}$	3.000	12.100
Lymphocytes	$/\mu\text{L}$	1.500	5.000
Monocytes	$/\mu\text{L}$	0	800
Eosinophil	$/\mu\text{L}$	0	1.300
Basophils	$/\mu\text{L}$	0	140
Platelet count	$\times 10^3/\mu\text{L}$	200	600
SERUM BIOCHEMISTRY			
Plasmatic protein	g/dL	5,3	7,6
Albumin	g/dL	2,3	3,8
ALT	U/L	10	88
Alkaline phosphatase	U/L	20	150
BUN	mg/dL	20	40
Creatinine	g/dL	0,7	1,4

*Mean corpuscular volume; ** Mean corpuscular hemoglobin; *** Mean corpuscular hemoglobin concentration. Source: Miotto, B.A.; 2016.

CHAPTER 3:

**Molecular and serological characterization of the first *Leptospira*
santarosai strain isolated from a dog**

3 MOLECULAR AND SEROLOGICAL CHARACTERIZATION OF THE FIRST LEPTOSPIRA SANTAROSAI STRAIN ISOLATED FROM A DOG

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4.1 INTRODUCTION

Leptospirosis is a zoonotic disease of global importance caused by pathogenic *Leptospira* species (BOURHY et al., 2014). It can affect a broad range of mammal species, and animals usually are subclinically infected, acting as reservoir hosts to particular *Leptospira* serovars (SCHULLER et al., 2015). These so-called maintenance hosts typically present non-symptomatic, highly intense and prolonged shedding of the pathogen via urine (Adler et al. 2011), thereby contributing to the spread of leptospires into the environment.

Pathogenic *Leptospira* are classified into more than 250 serovars and ten genomospecies (BOURHY et al., 2014). Dogs are referred as accidental hosts for many pathogenic *Leptospira*, and clinical leptospirosis is classically associated with *L. interrogans* and *L. kirschneri* infection (SCHULLER et al., 2015). Although dogs are considered maintenance hosts of *L. interrogans* serovar Canicola, the association of particular serovars with specific maintenance hosts may not be absolute, and leptospiuria in asymptomatic dogs can be attributed to other *L. interrogans* serovars, including strains from Sejroe serogroup (SCANZIANI et al., 1995; RÜHL-FEHLERT et al., 2000). There is no record to date of clinical leptospirosis or chronic carrier state attributed to *Leptospira santarosai* infection in dogs, either of serogroup Sejroe or any other serogroup.

Leptospira santarosai has been isolated particularly in Latin America (NALAM et al., 2010), and renal carriage of the pathogen is associated with different animal hosts (HAMOND et al., 2015). *L. santarosai* has also been isolated from human subjects presenting acute leptospirosis (BOURHY et al., 2013; VALVERDE et al., 2013; CHOU et al., 2014), highlighting the importance of animal reservoirs in the zoonotic transmission of this particular pathogen.

Herein we report the molecular and serological characterization of a *L. santarosai* strain isolated from a dog.

4.2 MATERIAL AND METHODS

4.2.1 Samples and study design

Blood and urine samples were taken from a dog kept in a public shelter at the University of São Paulo campus, located in São Paulo city, Brazil. Screening for dogs presenting persistent urinary shedding of leptospires was carried out between July of 2013 and January of 2014 as part of a program to prevent canine leptospirosis at the shelter facility.

Blood was collected from jugular or cephalic vein and drawn into Venosafe® tubes containing EDTA (Terumo) and BD Vacutainer tubes (BD Diagnostics). Whole-blood was used for PCR testing and serum was recovered after sample clotting and centrifugation for biochemistry analysis and microscopic agglutination testing (MAT). Urine samples were taken by sterile urinary cystocentesis and used for PCR testing.

Prospective evaluation was performed to confirm persistence of infection; reevaluations included blood and urine PCR testing, leptospiral culture from urine samples and detection of anti-*Leptospira* sp. antibodies by MAT. Physical examination, serum biochemistry analysis and evaluation of hematologic parameters were also performed to exclude acute leptospiral infection. The isolated strain was characterized by molecular and serological techniques. This study was approved by the Ethical Committee of the School of Veterinary Medicine of the University of São Paulo (protocol 2706/2012).

4.2.2 Microscopic agglutination test

Anti-*Leptospira* sp. antibodies titers were determined by MAT using representative antigens from 18 serogroups following previous recommendation (World Organization for Animal Health, 2012). Endpoint titers were determined by starting at an initial dilution of 1:100 and using two-fold dilutions until the last well showing 50% agglutination was recorded. The cutoff for a positive agglutination reaction was defined as a titer ≥ 100 in a single sample.

4.2.3 *Leptospira* molecular detection

DNA from urine and blood samples was extracted using NucliSens miniMAG kit (BioMerieux, Inc., Durham, NC) according to manufacturer's instructions. Extracted DNA was subjected to PCR amplification using previously reported *Leptospira* genus-specific protocol targeting a 331bp fragment of the 16S rRNA gene (MÉRIEN et al., 1992). PCR amplification was carried out with one cycle at 94°C for 5min, 40 cycles at 94°C for 30s, 60°C for 30s, 72°C for 30s and a final extension at 72°C for 5min. *L. interrogans* sv. Canicola genomic DNA was used as positive control and DNAase-free water as negative control in all PCR runs. Amplified products were separated on a 2% agarose gel stained with SYBRSafe DNA Gel Stain (Invitrogen, Carlsbad, CA, EUA) to further observation using UV transillumination.

4.2.4 Isolation of leptospires

For leptospires recovery, 0.5 ml aliquots of urine samples were diluted in sterile physiological solution to a final concentration of 1:10 and 1:100 to further inoculation in semi-solid Fletcher and liquid EMJH medium (Difco Laboratories, Franklin Lakes, NJ, USA). Tubes were incubated at 28°C for 8 weeks and examined weekly by dark-field microscopy.

4.2.5 Serological characterization of the isolated strain

The isolate was serogrouped using a panel of 32 specific antisera according to previous recommendation (DIKKEN; KMETY, 1978). Serogroup identification was confirmed by observing the highest titration against the representative serovars included in the panel.

4.2.6 Molecular characterization of the isolated strain

DNA extracted from the isolated strain was subjected to partial *secY* and 16S rRNA gene amplification as previously described (MÉRIEN et al., 1992; AHMED et al., 2006). The amplicons were sequenced and sequences were compared to reference strains deposited on GenBank using the BLAST tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multilocus Sequence Typing analysis (MLST) was performed following previous recommendation (BOONSILP et al., 2011), and a phylogenetic tree from MLST concatenated sequences was constructed by BioNumerics 7.5 (Applied Maths NV).

4.3 RESULTS

Persistent leptospiral infection was confirmed in the evaluated dog. The dog (male, adult, unvaccinated) was examined once a week, for 14 weeks. No abnormalities in hematological or serum biochemistry parameters were observed, and the dog did not present signs of acute leptospirosis or positive PCR results from blood samples during the study period. Although the dog was seronegative throughout all evaluations, PCR tested positive in all urine samples and leptospires were recovered from two different occasions.

One of the isolated spirochaetes was selected to further serological and molecular characterization. Serogrouping showed a strong and specific reaction against serovars from Sejroe serogroup (12.800 for Guaricura, Hardjoprajitno and Wolffii serovars and 3.400 for Hardjobovis serovar), thus confirming serogroup identification of the isolated strain.

The BLAST analysis of 16S rRNA and *secY* sequences amplified from the isolated strain revealed 98% identity for *secY* and 99.7% for 16S rRNA with *L. santarosai* sequences available in GenBank (accession nos. KF311106.1; DQ882866.1; AY631883; AY996805). Both obtained sequences were submitted to GenBank (accession nos. KU682051; KU682052).

The MLST analysis revealed a new allelic profile (41/50/49/48/54/44/43), and therefore, a new sequence type (ST218). The new ST218 is a single locus variation from ST177 that belongs to the Brazilian *L. santarosai* serogroup Javanica serovar Fluminense

strain Aa3. A Maximum-Likelihood tree was constructed from the concatenated sequences of the MLST loci and clustered the new ST218 together with other *L. santarosai* STs (APPENDIX C - Figure 1), with higher proximity with ST177 and ST179, both Brazilian strains isolated from mouse and bovine species, respectively.

4.4 DISCUSSION

Here we report the molecular and serological characterization of the first *Leptospira santarosai* isolated from a dog. The strain was isolated from a dog presenting persistent asymptomatic infection confirmed by PCR. The identification of such individuals is crucial to manage leptospiral spread into the environment, and PCR has been successfully used to identify leptospiruric dogs in different locations (ROJAS et al., 2010; KOIZUMI et al., 2013; CHETTA, 2014; GAY; SOUPÉ-GILBERT; GOARANT, 2014; SAMIR et al., 2015). 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 as a screening test combined with further prospective evaluation of the infected animals may increase the chances of recovering leptospires in culture media for appropriate characterization; it can also provide clinical, laboratorial and serological data in order to fully characterize the dog's carrier status. In this study, prospective evaluation enabled the characterization of chronic leptospiral infection, as well as the isolation of leptospires from two different occasions. The absence of antibody titers and clinical/laboratorial abnormalities throughout the reevaluations consistently confirmed the asymptomatic long-term carriage of the isolated strain in the infected dog.

The molecular and serological characterization enabled the identification of the infecting strain as *Leptospira santarosai* serogroup Sejroe. Although the MLST analysis presented high sequence similarity with other Brazilian *L. santarosai* strains, the allelic profile observed indicates a new strain circulating in Brazil and suggest relative genetic heterogeneity of Brazilian *L. santarosai* strains.

Leptospira santarosai has been isolated from a wide range of mammalian species, such as rodents, raccoons, goats, bovines and water buffaloes (HAMOND et al., 2015), possibly reflecting highly effective mechanisms to persistently infect distinct animal reservoirs. Nonetheless, the mechanisms of leptospiral host adaptation are still a subject of debate and the molecular basis behind host interactions with particular serovars remain unclear (ADLER et al., 2011). Canine renal carriage of leptospires is usually attributed to *L. interrogans* infection, notably serovar Canicola (YASUDA et al., 1980; SAMIR et al., 2015). However, dogs were previously identified carrying *L. wolffii* (ZAKERI et al., 2010), and asymptomatic infection caused by *L. kirschneri* was recently reported (DA CUNHA et al., 2016), reinforcing our findings that dogs can harbour leptospires other than *L. interrogans*.

To the best of our knowledge, this is the first report of a *L. santarosai* infection in a dog. Isolation and proper characterization of leptospires remain crucial bottlenecks to access the role of particular *Leptospira* strains in the epidemiology of leptospirosis. The findings reported here also supplement better understanding of *L. santarosai* chain of transmission and may promote improved control and prevention strategies. Despite these results, the role of *L. santarosai* infection in dogs remains poorly understood and requires further investigation.

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APPENDIX C

Figure 1: Maximum-Likelihood tree based on the concatenated sequences of the seven loci for the 218 available STs

Source: <http://dx.doi.org/10.1016/j.actatropica.2016.06.007>

CHAPTER 4:
**Canine renal carriage of *Leptospira santarosai*, an unusual pathogen
affecting dogs**

5 CANINE RENAL CARRIAGE OF LEPTOSPIRA SANTAROSAI, AN UNUSUAL PATHOGEN AFFECTING DOGS

Ready for submission

5.1 INTRODUCTION

Leptospirosis is a worldwide spread zoonotic disease that can affect virtually all mammals (ADLER et al., 2011). It is characterized by a wide range of clinical symptoms, varying from severe and sometimes fatal outcomes to chronic and asymptomatic infections (ANDRE-FONTAINE, 2006). Pathogenic *Leptospira* are currently classified into more than 260 serovars and 10 pathogenic species (BOURHY et al., 2014), and each serovar is adapted to persistently infect kidney tissues of one or more animal species, thus establishing a co-evolutionary link with its host (ADLER et al., 2011). Maintenance hosts typically present asymptomatic, long-term and highly intense leptospiral shedding in the urine (SCHULLER et al., 2015), and identification as well as proper management of such individuals is crucial to prevent leptospiral spread into the environment.

Canine leptospirosis is frequently reported worldwide (AZOCAR-AEDO, 2015; SCHULLER et al., 2015) and dogs can be highly exposed to pathogenic *Leptospira* in urban settings, particularly stray and sheltered dog populations (SCANZIANI et al., 2002; GAY; SOUPÉ-GILBERT; GOARANT, 2014). Although susceptible to many serovars, dogs are considered maintenance hosts for *L. interrogans* serovar Canicola (SCHULLER et al., 2015), and despite chronic infection is indeed frequently associated with this serovar (ANDRE-FONTAINE, 2006), other *L. interrogans* serovars, such as Copenhageni, Pomona, Sejroe and Tarassovi can also be recovered from asymptomatic dogs (MACKINTOSH; BLACKMORE; MARSHALL, 1980; RÜHL-FEHLERT et al., 2000; SUEPAUL et al., 2009; GAY; SOUPÉ-GILBERT; GOARANT, 2014). Our group has recently described the characterization of the first *Leptospira santarosai* strain ever isolated from a dog (SEE CHAPTER THREE), demonstrating that dogs can also persistently harbour species other than *L. interrogans*.

Asymptomatic urinary shedding of *L. santarosai* can be observed in a wide range of reservoir hosts (HAMOND et al., 2015a), and the isolation of *L. santarosai* has also been reported in human cases of leptospirosis (BOURHY et al., 2013; VALVERDE et al., 2013; CHOU et al., 2014b), highlighting the importance of animal reservoirs in the zoonotic transmission of this particular pathogen. In the present study, we report the identification of stray and sheltered dogs presenting asymptomatic urinary shedding of different *Leptospira* sp., notably *L. santarosai*, an unusual leptospiral species affecting dogs.

5.2 MATERIAL AND METHODS

5.2.1 Study design and samples

In order to characterize chronic carriers of leptospires, blood and urine samples were taken from two distinct and apparently healthy dog populations: 24 stray dogs kept in a public shelter from the city of Mogi das Cruzes, located in the eastern region of São Paulo State, Brazil, in July of 2013, and seven stray dogs living inside the University of São Paulo (USP) campus, located in the west region of São Paulo city, Brazil, between January and June of 2013. Whole-blood was used for PCR testing and serum was recovered after sample clotting and centrifugation for biochemistry analysis and microscopic agglutination testing (MAT).

Urine samples were taken via sterile urinary catheterization or cystocentesis and used for PCR testing. Prospective reevaluations were performed only in dogs presenting leptospires in urine samples, and included blood and urine PCR testing, leptospiral culture from urine samples and anti-*Leptospira* sp. antibody titration. Physical examination and serum biochemistry analysis were also performed to exclude acute leptospirosis. This study was approved by the Ethical Committee of the College of Veterinary Medicine and Zootechny, University of São Paulo (Protocol 2706/2012).

5.2.2 Microscopic agglutination test (MAT)

Presence of anti-*Leptospira* sp. antibodies were determined by MAT using a panel of polyclonal rabbit antisera of 22 pathogenic reference serovars representing 18 known serogroups (provided by Royal Tropical Institute - KIT, Amsterdam). The cutoff for a positive agglutination reaction was defined as a titer ≥ 100 in a single sample.

5.2.3 Isolation of leptospires

Blood and urine samples (0.5 ml) were diluted in sterile physiological solution (1:10 and 1:100), and 0.5 ml of each solution was added to Fletcher and EMJH medium (BD Difco™). Samples were cultivated at 28°C for 12 weeks and examined weekly by dark-field microscopy for the presence of spirochaetes.

5.2.4 DNA extraction and PCR

DNA was extracted using NucliSens® miniMAG™ (BioMérieux Inc., Durham, NC, USA) according to manufacturer's instructions, with slight modifications; 1 ml of resuspended solution was used in the initial lysis step and the final elution step was performed with 40µL. Extracted DNA was subjected to PCR amplification using previously reported *Leptospira* genus-specific protocol targeting a 331bp fragment of 16S rRNA gene (MÉRIEN et al., 1992). Samples presenting positive yields were also subjected to a partial *secY* amplification as previously described (AHMED et al., 2006).

5.2.5 Sequencing and phylogenetic analysis

Amplicons were sequenced on an ABI 7500 Genetic Analyzer (Life Technologies, Waltham, MA, USA) and edited using BIOEDIT Sequence Alignment Editor 7.0.9 (Hall, 1999 - Ibis Biosciences, Carlsbad, CA, USA). Sequences were compared to the GenBank nucleotide non-redundant database through BLAST analysis and a phylogenetic tree was constructed using Mega 5.10 software (TAMURA et al., 2013); the Maximum-Likelihood method with Tamura-Nei model and Nearest Neighbor Interchanges (1000 bootstrap replicates) were used for branch support statistical inference.

5.3 RESULTS

From the 24 dogs kept at the public shelter, one dog (dog A) presented urinary shedding of leptospires detected by PCR (4.1%), with no concomitant serum titers against *Leptospira* and no record of previous immunization. From the seven stray dogs living at the USP campus, two presented leptospiral urinary shedding detected by PCR (28.5%). One of the dogs (dog B) had serum titers of 400 against Hardjo and Wolffii serovars (Sejroe serogroup) and unknown immunization record; the other leptospiruric dog (dog C) had serum titers of 200 against serovar Grippothyphosa (serogroup Grippothyphosa) and had been immunized against leptospirosis (Recombitec™ C6/CV, Merial Inc.) by local caretakers approximately 6 months prior to the sample collection.

Dog A was euthanized a few days after the first sample collection due to clinical complications attributed to a vaginal neoplasia and dog B could not be located again after the first evaluation, sidelining prospective evaluations. Dog C was examined four times throughout a 12-week period. The dog presented positive PCR results in all urine samples; however, it was not possible to recover leptospires in culture media. No PCR amplification from blood samples or clinical abnormalities related to acute leptospirosis were observed in any of the dogs included in this study. PCR results, serological data, laboratorial findings and immunization status of all leptospiruric dogs are presented in Table 1 (APPENDIX D).

The 16S rRNA sequence analysis enabled the identification of *L. santarosai* species infecting two dogs (dogs A and B), with 99.5% identity with *L. santarosai* serovar Shermani strain LT821 ATCC43286 (AY631883), whereas the *Leptospira* DNA recovered from dog C

presented 99.6% of sequence identity with *L. interrogans* serovar Canicola strain Hond Utrecht IV (AY996798). The *secY* analysis also confirmed the identification of *L. santarosai* in dogs A and B, with >99% identity with other Brazilian *L. santarosai* strains (EU357994, KF366254, KP862646, KP862645, KP862632), and Dog C presented 99.6% of sequence identity with *L. interrogans* serovar Canicola strain Hond Utrecht IV (EU357961).

For *secY* phylogenetic analysis, the sequence of a previously described canine Brazilian *L. santarosai* strain DU92 (KU682052) was included in the alignment, as well as other publically available sequences of Brazilian *L. santarosai* recovered from different host species. The *secY* analysis enabled not only the distinction of *L. interrogans* and *L. santarosai* among the leptospiruric dogs, but also highlighted the clustering of the Brazilian *L. santarosai* obtained from dogs (Figure 1 – APPENDIX D). Both *secY* and 16S rRNA sequences obtained from dogs infected by *L. santarosai* presented high identity when compared to sequences from the DU92 strain (Supplementary material 1 – APPENDIX D). All obtained sequences were deposited in GenBank and the accession numbers can be visualized in Table 1.

5.4 DISCUSSION

PCR has been successfully used to characterize urinary shedding of leptospires and recent reports using PCR have identified leptospiruric dogs in different locations (ROJAS et al., 2010; ZAKERI et al., 2010; GAY; SOUPÉ-GILBERT; GOARANT, 2014; SAMIR et al., 2015). In this study, PCR was able to identify urinary shedding of leptospires in both populations studied. Prospective evaluation confirmed persistent and asymptomatic renal carriage of *Leptospira* in one of the three leptospiruric dogs (dog C); However, it was not possible to recover leptospires in urine samples from dog C, even those with positive PCR results, evidencing the difficulties of growing leptospires in culture media.

Although sequencing PCR products can potentially endorse species identification and promote better understanding of the local disease epidemiology (ZAKERI et al., 2010), most studies investigating leptospiruric dogs use PCR based-techniques predominantly as a diagnostic tool, with no regards to the identification of the infecting species. Sequence

analysis enabled the identification of two distinct *Leptospira* species infecting dogs in this study. One of the dogs (dog C) presented *L. interrogans* infection, and the other two leptospiruric dogs (dogs A and B) presented asymptomatic infection caused by *L. santarosai*. Only recently *L. santarosai* infection was firstly described infecting the canine species (SEE CHAPTER THREE); the isolated strain (strain DU92) was serologically characterized as belonging to Sejroe serogroup and was recovered from an asymptomatic dog living at a shelter facility located inside the USP campus. Curiously, one of the dogs infected by *L. santarosai* reported here (dog B) was also captured at the USP campus and presented serum titers exclusively against Sejroe serogroup, possibly indicating infection caused by a local *L. santarosai* strain circulating around the university campus. Even though the canine sequences (DU01/96, DUPA and DU92) presented some level of identity with Brazilian *L. santarosai* strains isolated from bovine, including the reference serovar Guaricura strain Bov.G, they appear to cluster separately. Moreover, all *secY* and 16S rRNA sequences obtained from dogs infected by *L. santarosai* (including strain DU92) presented high identity among themselves, suggesting a possible genetic distinction between lineages of Brazilian *L. santarosai* maintained by dogs and other animal hosts.

Asymptomatic renal carriage of *L. santarosai* is typically observed in wild animals, such as raccoons and wild rodents (see Leptospirosis Reference Center, Institute Pasteur, France, <http://www.pasteur.fr/recherche/Leptospira/LeptospiraF.html>). Interestingly, it has also been isolated from different domestic species, such as goats (LILENBAUM et al., 2014), cattle (HAMOND et al., 2015b) and water buffaloes (VASCONCELLOS et al., 2001), particularly in Latin America. Recent whole-genome sequencing of *L. santarosai* revealed genomic regions encoding transposases and hypothetical proteins that may enhance fitness and might play an important role in the *L. santarosai* pathogenesis (CHOU et al., 2014a). Our results reinforce the ability of *L. santarosai* to successfully colonize renal tubules of distinct host species.

The identification of two more dogs from distinct populations presenting asymptomatic *L. santarosai* infection reported here highlights a possible and unexpected role of dogs in the chain of transmission of this particular pathogen in urban environments. It also indicates that canine renal carriage of this leptospiral species may not be merely an occasional finding in Brazil. Although the role of dogs in the transmission of leptospires in urban settings is not clearly established, knowledge of circulating leptospires and their

reservoirs is essential to implement effective diagnostic strategies and successful control programs, and the role of *L. santarosai* infection in dogs needs further investigation.

ACKNOWLEDGMENTS

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APPENDIX D

Table 1: Description of all available data from dogs presenting leptospirosis according to each evaluation performed, São Paulo, Brazil.

Dog	Evaluation date	Immunization status	Renal function		PCR		Culture		MAT					Phylogenetic analysis		
			BUN (mg/dL)	creat. (mg/dL)	blood	urine	urine	GRI	PO	BU	HA	WO	species identity	16S rRNA (access. nº)	secY (access. nº)	
A	01.20.13	unknown	22.4	0.58	(-)	(+)	NP	(-)	(-)	(-)	(-)	(-)	<i>L. santarosai</i>	KX026948	KX008569	
B	01.20.13	unknown	27.1	0.80	(-)	(+)	NP	(-)	(-)	(-)	400	400	<i>L. santarosai</i>	KX008567	KX008567	
C	01.20.13	immunized	21.8	0.65	(-)	(+)	NP	200	(-)	(-)	(-)	(-)	<i>L. interrogans</i>	KX008569	KX026950	
C	02.04.13		24.1	0.71	(-)	(+)	(-)	100	200	200	(-)	(-)				
C	03.10.13		23.8	0.66	(-)	(+)	(-)	100	(-)	(-)	(-)	(-)				
C	04.22.13		23.3	0.67	(-)	(+)	(-)	200	100	400	(-)	(-)				

(-): negative; (+); positive; BUN: blood urea concentration; creat.: creatinine GRI: Grippotyphosa; BU: Butembo; HA: Hardjo (hardjoprajitno); WO: Wolffii. NP: not performed. Source: Miotto, B.A.; 2016.

Figure 1: Maximum-Likelihood tree constructed with Tamura-Nei model and Nearest Neighbor Interchanges with 1000 bootstrap replicates based on the *secY* sequences of *Leptospira* DNA recovered from leptospiruric dogs and reference strains. The red branches feature sequences of Brazilian *L. santarosai* strains used for the comparative analysis.

Source: Miotto, B. A. (2016).

Supplementary material 1: *Leptospira santarosai* strains *secY* sequences identity (%).

<i>L. santarosai</i> serovar Navet str. TRVL109873 - EU358067	ID											
<i>L. santarosai</i> serovar Rama str. 316 - EU358063	0.98	ID										
<i>L. santarosai</i> serovar Canalzonae str. CZ188 - EU358029	0.97	0.98	ID									
<i>L. santarosai</i> serovar Guaricura str. BovG - EU357994	0.99	0.98	0.98	ID								
<i>L. santarosai</i> str. Carioca isolate M72/06-13 - KF311106	0.96	0.96	0.96	0.98	ID							
<i>L. santarosai</i> str. Bananal - KF366254	0.98	0.97	0.97	0.98	0.96	ID						
<i>L. santarosai</i> str. 2013_U280 - KP862646	0.96	0.96	0.96	0.97	1.00	0.96	ID					
<i>L. santarosai</i> str. 2013_U278 - KP862645	0.97	0.97	0.97	0.97	0.96	0.99	0.96	ID				
<i>L. santarosai</i> str. 2013_U152 - KP862632	0.97	0.97	0.97	0.97	0.96	0.99	0.96	1	ID			
DU92	0.95	0.94	0.95	0.96	0.98	0.95	0.98	0.96	0.96	ID		
DU01/96 - Dog A	0.95	0.94	0.95	0.96	0.98	0.95	0.98	0.96	0.96	1	ID	
DUPA - Dog B	0.95	0.94	0.95	0.96	0.98	0.95	0.98	0.96	0.96	1	1	ID

6 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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