

**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS**

**LEPTOSPIROSE CANINA: DADOS CLÍNICOS, LABORATORIAIS E
TERAPÊUTICOS EM CÃES NATURALMENTE INFECTADOS**

SIMONE TOSTES DE OLIVEIRA

PORTO ALEGRE

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TERAPÊUTICOS EM CÃES NATURALMENTE INFECTADOS**

Autora: Simone Tostes de Oliveira

Tese apresentada como requisito parcial para
obtenção do grau de Doutor em Ciências
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Patologia Animal

Orientador: Félix Hilario Diaz González

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1. Doenças infecciosas dos animais: cães 2. Leptospirose: cães

Simone Tostes de Oliveira

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CÃES NATURALMENTE INFECTADOS.

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EPÍGRAFE

História natural

*“Cobras cegas são notívagas.
O orangotango é profundamente solitário.
Macacos também preferem o isolamento.
Certas árvores frutificam de 25 em 25 anos.
Andorinhas copulam no vôo.
O mundo não é o que pensamos.”*

Carlos Drummond de Andrade

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RESUMO

A leptospirose é uma zoonose de ampla distribuição mundial, causada pela infecção por sorovares patogênicos do gênero *Leptospira*. Assim como outras espécies de animais acometidos, os cães que sobreviverem à fase aguda da doença podem se tornar portadores, excretando a bactéria através da urina. Algumas alterações no hemograma, bioquímica sérica e urinálise, juntamente com o histórico do paciente e fatores de risco, auxiliam na suspeita da doença; porém, o diagnóstico definitivo é realizado através de testes mais específicos. Estes testes incluem sorologia, técnicas moleculares como a reação em cadeia da polimerase (PCR) e cultura para isolamento do sorovar. O presente trabalho caracterizou e comparou a leptospirose em três populações caninas de Porto Alegre. Foram avaliados 33 cães com suspeita da doença, atendidos no Hospital de Clínicas Veterinárias (HCV) da Universidade Federal do Rio Grande do Sul (UFRGS); 65 cães provenientes do Centro de Controle de Zoonoses (CCZ) de Porto Alegre; e 155 cães residentes no bairro Arquipélago na zona urbana de Porto Alegre, onde existe uma alta incidência de leptospirose humana. O diagnóstico da leptospirose canina foi baseado na sorologia e PCR no soro e urina. Um total de 14,6% (37/253) dos cães apresentaram resultado positivo na PCR no sangue (leptospiremia) e 14,2 % (36/253) na urina (leptospirúria). Em relação à sorologia, 48,2% (122/253) foram positivos para um ou mais sorovares. Os sorovares mais prevalentes foram canicola, icterohaemorrhagiae e copenhageni. A presença de ratos no ambiente foi associada a leptospirúria ($P=0,02$). Na população do HCV, o aumento da creatinina sérica ($P=0,009$), icterícia ($P=0,004$) e glicosúria ($P=0,04$) foram associados com leptospirúria. Apesar destas associações encontradas, observou-se que a ausência de sinais clínicos ou de alterações no hemograma, bioquímica sérica ou urinálise não excluíram a infecção ($P>0,05$). Em um segundo estudo, foi investigada a eficácia da doxiciclina na eliminação do estado de portador renal em quatro cães assintomáticos. Destes, três estavam infectados com o sorogrupo Canicola e um com o sorogrupo Icterohaemorrhagiae. Os cães foram acompanhados por 30 dias após o início do tratamento, e a ausência de leptospiras na urina foi confirmada através de três resultados seriados de PCR negativa em cada cão. Finalmente, valor preditivo da proteína C-

reativa (C-RP) na leptospirose foi investigado em 62 cães, comparando sua concentração sérica e urinária com os resultados de sorologia e PCR. Sorologia positiva foi associada com C-RP urinária (P= 0,038). Houve apenas associação fraca entre proteína C-reativa sérica e PCR no sangue (área sob a curva= 0,68), e não foi observada associação entre C-RP urinária e PCR na urina. Não foram observadas vantagens de se incluir a C-RP como um teste de triagem para leptospirose em cães. As informações obtidas com os estudos aqui citados mostra a importância do diagnóstico definitivo, preferencialmente realizado através de PCR; a necessidade de se testar cães expostos a fatores de risco, independente de seu estado aparente de saúde; a importância de medidas sanitárias para a prevenção da doença e o tratamento adequado para que se elimine o possível estado de portador renal dos cães.

Palavras-chave: *Leptospira*, cão, diagnóstico, PCR, sorologia, tratamento, proteína C-reativa.

Canine leptospirosis: clinical, laboratorial and therapeutical data in naturally infected dogs

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ABSTRACT

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic serovars of the genus Leptospira. As other affected species of animals, the dogs that survive the acute phase of the disease can become carriers, excreting the bacteria in the urine. Some alterations in blood count, serum biochemistry and urinalysis, along with patient history and risk factors, may contribute to a presumptive diagnosis, but the definitive diagnosis is made using more specific tests. These tests include serology, molecular techniques like polymerase chain reaction (PCR) and culture for the serovar isolation. The present study characterized and compared leptospirosis in three canine populations of Porto Alegre, RS. Thirty three dogs with suspected disease were evaluated at the Veterinary Hospital of the Federal University of Rio Grande do Sul, 65 dogs from the Control Center of Zoonoses from Porto Alegre and 155 dogs from Archipelago neighborhood, where there is a high incidence of human leptospirosis. The diagnosis of canine leptospirosis was based on serology and PCR in serum and urine. A total of 14.6% (37/253) of dogs tested positive in PCR in blood (leptospiremia) and 14.2% (36/253) in the urine (leptospiruria). With regard to serology, 48.2% (122/253) were positive for one or more serovars. The most prevalent serovars were canicola, icterohaemorrhagiae and copenhageni. The presence of rats in the environment was associated with leptospiruria ($P = 0.02$). In the Veterinary Hospital population, increased serum creatinine ($P = 0.009$), jaundice ($P = 0.004$) and glucosuria ($P = 0.04$) were associated with leptospiruria. Despite these associations, it was observed that the absence of clinical signs or changes in blood count, serum biochemistry and urinalysis did not exclude infection ($P > 0.05$). In a second study, we investigated the effectiveness of doxycycline to eliminate the carrier state in four asymptomatic dogs. Three were infected with serogroup Canicola and one with serogroup Icterohaemorrhagiae. The dogs were followed for 30 days after starting treatment, and the absence of leptospire in urine was confirmed by three serial results of negative PCR in each dog. Finally, the predictive value of C-reactive protein (C-RP) in leptospirosis was investigated in 62 dogs, comparing its serum and urinary concentrations with serology and PCR.

Seropositivity was associated with urinary C-RP ($P = 0.038$). There was only a weak association between C-RP and serum PCR in blood ($AUC = 0.68$), and no association was found between urinary C-RP and PCR in urine. There were no advantages to include the C-RP as a screening test for leptospirosis in dogs. The information gathered from the studies cited here shows the importance of the definitive diagnosis, preferably performed by PCR; the need to test dogs exposed to risk factors, regardless of their apparent health condition, the importance of sanitary surveillance for the prevention of the disease, and adequate treatment to eliminate the carrier state.

Key words: *Leptospira, dog, diagnosis, PCR, serology, treatment, C-reactive protein.*

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1. INTRODUÇÃO

A leptospirose é causada por bactérias patogênicas do gênero *Leptospira* e acomete diversas espécies animais, incluindo os humanos. Tem distribuição cosmopolita, sendo mais prevalente em países de clima tropical. A transmissão se dá pelo contato direto com animais infectados ou sua urina, ou pelo contato indireto via solo ou água contaminados. Nos animais portadores, as leptospiras se localizam nos túbulos renais e são excretadas na urina por um período de tempo variável. Os roedores são considerados a principal fonte de transmissão da doença, porém, os cães, por sua proximidade com os humanos, também apresentam alto potencial zoonótico.

Estudos sorológicos em estados do sul do Brasil mostraram grande incidência de leptospirose tanto em cães quanto em animais de produção, como bovinos, caprinos e suínos. A incidência da leptospirose humana no Rio Grande do Sul é elevada, se comparada à média do país. Em Porto Alegre, o número de casos por 100.000 habitantes variou entre 0,85 e 7,14 entre 1996 e 2007. Em um estudo realizado em Porto Alegre, entre os anos de 2001 e 2006, verificou-se que as pessoas que residiam em bairros compreendidos pelo estrato socioeconômico baixo tinham 5 vezes mais chance de se expor a ambientes contaminados, se comparadas com as residentes em bairros do estrato socioeconômico alto. O mesmo estudo mostrou que o número de doentes foi significativamente maior em locais onde havia roedores no ambiente, entulho, e saneamento básico precário.

Dois estudos em cães foram realizados no Hospital de Clínicas Veterinárias da UFRGS, em Porto Alegre, baseados no diagnóstico sorológico para leptospirose. Um dos estudos (SANTIN et al., 2006) avaliou um grupo de cães suspeitos e um grupo controle, verificando a mesma quantidade de positivos (37%) em ambos os grupos. Outro trabalho (DALMOLIN & GONZÁLEZ, 2007), realizado posteriormente, avaliou a porcentagem de positivos dentre cães suspeitos de leptospirose e identificou 67% de positivos. No primeiro trabalho, o fato do grupo suspeito apresentar a mesma porcentagem de positivos em relação ao grupo controle, poderia sugerir a presença de animais assintomáticos, enquanto que no segundo estudo, a maior porcentagem de cães positivos, em relação ao primeiro trabalho, poderia sugerir um aumento do número de casos. Apesar de sua importância, estudos baseados apenas em sorologia devem ser avaliados com alguma cautela, visto haver algumas limitações da técnica. Os anticorpos só são

detectáveis após 5-10 dias do início da infecção; alguns animais apresentarão níveis de anticorpos por meses ou anos após o contato com o agente, não indicando necessariamente uma infecção ativa; a sorologia não permite distinguir títulos por vacina ou pela doença naturalmente adquirida; alguns animais portadores podem apresentar títulos abaixo dos níveis detectáveis, sendo portanto, falso-negativos. Apesar de que técnicas que detectem a presença de IgM na amostra poderiam ser úteis para elucidar se a infecção é recente ou não, como utilizado em humanos (ELISA IgM), o teste de rotina disponível para cães é o teste de aglutinação microscópica (MAT), o qual não permite a diferenciação de IgM e IgG. Assim sendo, em cães é importante que se associe outras técnicas diagnósticas, como por exemplo a detecção da presença de leptospiras no sangue e urina através da reação em cadeia da polimerase (PCR). Porém, pelo fato da PCR ainda não estar amplamente disponível como exame de rotina, também é útil a investigação de alterações, clínicas ou clinicopatológicas, que sugiram que um cão encontre-se infectado, tanto na fase aguda quanto no papel de portador renal. Desta forma, estes exames podem direcionar o clínico, para que este solicite os exames mais específicos para o diagnóstico definitivo.

A manifestação da leptospirose em cães é variável, podendo ser clínica ou subclínica. Os animais que sobreviverem à fase aguda da doença, de leptospiremia, podem, em um segundo estágio, tornar-se carreadores assintomáticos, através da excreção urinária de leptospiras no meio ambiente. A fase crônica da doença, que se inicia por volta da segunda semana após o contato com o agente, geralmente é acompanhada da produção de anticorpos e leptospirúria. Apesar de que algumas alterações clinicopatológicas sejam esperadas, principalmente no que se refere a alterações renais e hepáticas, resultados normais de hemograma, bioquímica sérica e urinálise não excluem o diagnóstico de leptospirose. Ainda assim, estes exames podem ser de grande auxílio para o prognóstico do paciente e acompanhamento da progressão ou regressão de lesões, contribuindo para a decisão das medidas mais adequadas a serem tomadas quanto ao tratamento.

Estudos *in vitro* e *in vivo* são descritos para testar a eficácia dos antibióticos no tratamento da leptospirose, sendo o hamster o modelo animal experimental mais utilizado. Vários antibióticos melhoram os sinais clínicos do paciente, sem, no entanto, eliminar as leptospiras do rim e da urina, causando na verdade, um grande problema quando não se obtém o diagnóstico da doença, permanecendo o animal muitas vezes no estado de portador renal e sendo fonte de infecção da leptospirose. A doxiciclina e a estreptomicina, em condições experimentais

controladas, foram eficazes para eliminar a leptospira do tecido renal. É importante que sejam realizados estudos para avaliar a eficácia do tratamento por um tempo mais prolongado em cães com leptospirúria naturalmente infectados, pois os sorovares encontrados no ambiente podem apresentar características e comportamento diferentes das cepas mantidas em laboratório, em relação a virulência e resistência. A caracterização da leptospirose em diferentes populações de cães, assim como a comparação entre elas, auxilia no entendimento da doença e possíveis medidas de prevenção e tratamento.

2 OBJETIVOS

2.1 Objetivo geral

Fundamentar a preditividade de métodos diagnósticos na leptospirose canina para apoiar o clínico de pequenos animais no diagnóstico e tratamento da doença em cães, trazendo benefícios tanto na área de medicina preventiva quanto no bem-estar animal.

2.2 Objetivos específicos

- Caracterizar a infecção por *Leptospira* em três populações de cães em Porto Alegre;
- Identificar, nestes cães, diferenças quanto aos sinais clínicos, fatores de risco e alterações clinicopatológicas produzidas por diferentes sorovares, com base na sorologia e técnicas moleculares;
- Verificar a presença de leptospiras na urina e no sangue de cães suspeitos de leptospirose, através da reação em cadeia da polimerase (PCR), e compará-la ao diagnóstico por titulação de anticorpos (teste sorológico de aglutinação microscópica, MAT);
- Realizar um estudo molecular das formas presentes nas amostras, quando possível, e compará-las com os isolados descritos na literatura;
- Comparar as genomoespécies presentes na urina com o diagnóstico sorológico.
- Verificar a eficácia da doxiciclina na eliminação da leptospirúria através do acompanhamento dos cães com diagnóstico positivo durante e após o tratamento;
- Verificar as alterações nos níveis da proteína C-reativa sérica e urinária em cães com leptospirose.

3. REVISÃO BIBLIOGRÁFICA

3.1. Histórico

A leptospirose foi descrita pela primeira vez em 1886 por Adolf Weil, como uma síndrome em pacientes humanos apresentando icterícia e insuficiência renal. No entanto, síndromes aparentemente idênticas foram relatadas anteriormente em diversos países e relacionadas com risco ocupacional, porém sem a identificação de um agente causador (LEVETT, 2001).

3.2. Classificação sorológica e genotípica

Na classificação sorológica, o gênero *Leptospira* é dividido em duas espécies, *L. interrogans*, englobando todas as cepas patogênicas, e *L. biflexa*, contendo as cepas saprófitas isoladas do ambiente. As duas espécies contêm vários sorovares, definidos por aglutinação de anticorpos. Os sorovares antigenicamente relacionados são agrupados em sorogrupos (LEVETT, 2001; MICHEL et al. 2002) (Tabela 1).

Na classificação genotípica, sendo esta mais recente que a sorológica, estão incluídos todos os sorovares, tanto da *L. interrogans* quanto da *L. biflexa* descritas anteriormente, porém as genomoespécies não correspondem às duas espécies previamente descritas, e, além disto, sorovares patogênicos e não patogênicos ocorrem dentro da mesma espécie (LEVETT, 2001) (Tabelas 2 e 3).

A coexistência das duas classificações é confusa, porque apesar de usar a mesma terminologia para os sorovares, elas não se sobrepõem. Até o momento, para estudos de diagnóstico experimental e epidemiológico, a classificação sorológica ainda é usada (MICHEL et al. 2002), porém, a reclassificação das leptospiros sob análise molecular do genoma é taxonomicamente correta e fornece uma forte base para futuras classificações (LEVETT, 2001).

Para melhor compreensão das diferenças entre as classificações, o sorogrupo Icterohaemorrhagiae foi marcado em cinza nas tabelas como exemplo. Sorologicamente, ele está classificado como sendo da espécie *L. Interrogans*, e a ele pertencem os sorogrupos icterohaemorrhagiae, copenhageni e lai. As espécies na classificação genotípica não

correspondem às espécies previamente descritas, apesar de duas delas (*L. interrogans* e *L. biflexa*, segundo a classificação sorológica) continuarem com o mesmo nome, o que torna o entendimento ainda mais confuso. Continuando com o exemplo do sorogrupo Icterohaemorrhagiae, este na classificação genotípica pertence a mais de uma espécie (*L. interrogans*, *L. kirschneri*, *L. weilii* e *L. inadai*), como mostrado nas Tabelas 2 e 3. Os sorovares pertencentes a cada sorogrupo não sofreram alterações, e a nomenclatura da relação sorogrupo-sorovar é a mesma, tanto na classificação sorológica quanto genotípica.

Tabela 1. Sorogrupos e alguns sorovares da espécie *L. interrogans* sensu lato (classificação sorológica).

Sorogrupo	Sorovar(es)
Icterohaemorrhagiae	icterohaemorrhagiae, copenhageni, lai
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, georgia
Tarassovi	tarassovi
Ballum	ballum, arborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

(Fonte: Levett, 2001).

Tabela 2. Espécies genômicas de *Leptospira* e distribuição dos sorogrupos (classificação genotípica).

Espécie	Sorogrupo (s)
<i>L. interrogans</i>	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
<i>L. noguchii</i>	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
<i>L. santarosai</i>	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
<i>L. meyeri</i>	Ranarum, Semarang, Sejroe, Mini, Javanica
<i>L. wolbachii</i>	Codice
<i>L. biflexa</i>	Semarang, Andamana
<i>L. fainei</i>	Hurstbridge
<i>L. borgpetersenii</i>	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
<i>L. kirschneri</i>	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
<i>L. weilii</i>	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
<i>L. inadai</i>	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
<i>L. parva</i>	Turneria
<i>L. alexanderi</i>	Manhao, Hebdomadis, Javanica, Mini

(Fonte: Levett, 2001).

Tabela 3. Espécies genotípicas associadas aos sorogrupos.

Sorogrupo	Espécies genotípicas
Andamana	<i>L. biflexa</i>
Australis	<i>L. interrogans, L. noguchii, L. borgpetersenii, L. kirschneri</i>
Autumnalis	<i>L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri</i>
Ballum	<i>L. borgpetersenii</i>
Bataviae	<i>L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri</i>
Canicola	<i>L. interrogans, L. inadai, L. kirschneri</i>
Celledoni	<i>L. weilii, L. borgpetersenii</i>
Codice	<i>L. wolbachii</i>
Cynopteri	<i>L. santarosai, L. kirschneri</i>
Djasiman	<i>L. interrogans, L. noguchii, L. kirschneri</i>
Grippotyphosa	<i>L. interrogans, L. santarosai, L. kirschneri</i>
Hebdomadis	<i>L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. kirschneri, L. alexanderi</i>
Hurstbridge	<i>L. fainei</i>
Icterohaemorrhagiae	<i>L. interrogans, L. weilii, L. inadai, L. kirschneri</i>
Javanica	<i>L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. inadai, L. alexanderi</i>
Louisiana	<i>L. interrogans, L. noguchii</i>
Lyme	<i>L. inadai</i>
Manhao	<i>L. weilii, L. inadai, L. alexanderi</i>
Mini	<i>L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. alexanderi</i>
Panama	<i>L. noguchii, L. inadai</i>
Pomona	<i>L. interrogans, L. noguchii, L. santarosai, L. kirschneri</i>
Pyrogenes	<i>L. interrogans, L. noguchii, L. weilii, L. santarosai, L. borgpetersenii</i>
Ranarum	<i>L. interrogans, L. meyeri</i>
Sarmin	<i>L. interrogans, L. weilii, L. santarosai</i>
Sejroe	<i>L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri</i>
Semaranga	<i>L. meyeri, L. biflexa</i>
Shermani	<i>L. noguchii, L. santarosai, L. inadai</i>
Tarassovi	<i>L. noguchii, L. weilii, L. santarosai, L. borgpetersenii, L. inadai</i>

Fonte: Levett (2001)

3.3. Fonte da transmissão da leptospirose e considerações sobre os cães

A prevalência da leptospirose em cães varia consideravelmente entre áreas e entre países, sendo mais elevada em regiões tropicais (JOUGLARD & BROD, 2000). As leptospiras podem se manter viáveis no ambiente por meses, sob condições de umidade e água parada ou com pouco movimento, e com pH neutro a levemente alcalino (LANGSTON & HEUTER, 2003). Os cães, como outras espécies de animais domésticos e silvestres, são susceptíveis a todos os sorogrupos de leptospira conhecidos. A leptospirose canina constitui um problema sanitário de grande importância, não somente pela gravidade de sua patogenia, mas também como elemento de contágio ao ser humano (JOUGLARD & BROD, 2000). Os cães são considerados hospedeiros reservatórios dos sorovares canicola e bataviae, e possivelmente do bratislava (LAPPIN, 2003). Como regra geral para todas as espécies, animais infectados com os sorovares de leptospira para os quais são adaptados podem excretar a bactéria persistentemente por toda a vida (HEATH & JOHNSON, 1994).

Cães são boas sentinelas para detectar a presença de *Leptospira* no ambiente e são fatores chave para o entendimento da ecologia da doença. Devido a sua relevância como animais de estimação, é muito importante o diagnóstico e tratamento da doença nestes animais (GHNEIM et al., 2007).

3.4. Fatores de risco e prevenção da leptospirose canina

São considerados fatores de risco à leptospirose a habitação em áreas periurbanas (WARD et al., 2004), a presença de roedores no domicílio, o hábito de se manter os cães com acesso à rua (JORGE et al., 2005), o contato dos cães com áreas alagadiças (JOUGLARD & BROD, 2000) e o consumo de carne crua (MEEYAM et al., 2006). Um estudo concluiu que cães mantidos em pátios abertos apresentaram risco duas vezes maior para contrair a doença, e a ausência de esgotos nas residências foram os principais fatores de risco à leptospirose (FURTADO et al., 1997). Jorge et al. (2005) ressaltaram a importância sobre o esclarecimento da população do local quanto à epidemiologia da leptospirose e a importância da prevenção, através de medidas sanitárias aplicadas ao ambiente e aos animais de estimação.

As leptospirosas são suscetíveis a detergentes, desinfetantes a base de iodóforos e desidratação. Material contaminado com urina deve ser manipulado como risco biológico. Nas clínicas e hospitais, cães suspeitos ou com diagnóstico confirmado não devem urinar em áreas frequentadas por outros cães. As gaiolas podem ser limpas com hipoclorito e papel toalha (LANGSTON & HEUTER, 2003).

3.5 A leptospirose no Rio Grande do Sul

O Rio Grande do Sul apresenta uma alta incidência de leptospirose humana, com cerca de 10 casos por 100 mil habitantes, superior à média do país, que é de 3,5 casos por 100 mil habitantes. Os municípios com maior número de casos estão localizados principalmente na região central e sul do estado. Os resultados encontrados sugerem a existência de características ecológicas favoráveis à transmissão da leptospirose em locais de proliferação de roedores sinantrópicos e de produção agrícola intensiva (BARCELLOS et al., 2003). Porto Alegre registrou 0,85 a 7,14 casos/100.000 habitantes entre os anos de 1996 e 2007 (THIESEN et al., 2008). Um estudo investigou os fatores de risco para leptospirose humana em Porto Alegre, e verificou-se que a presença de roedores e entulho e o contato com esgoto foram significativos. Verificou-se também que as pessoas residentes nos bairros de estrato socioeconômico baixo tinham cerca de 5 vezes mais chance de se expor a ambientes contaminados, em relação aos residentes em bairros de estrato socioeconômico alto (HENKES, 2008).

Foi verificada a presença de vários sorovares infectantes no Rio Grande do Sul, e sua prevalência variou de acordo com a espécie animal acometida. Um estudo sorológico foi realizado em caprinos leiteiros de 15 municípios do Rio Grande do Sul, sendo encontrado nos animais soropositivos os sorovares *icterohaemorrhagiae*, *pomona* e *hardjo* (SCHMIDT et al., 2002). Em um outro estudo com 1630 ovinos da região sudeste e sudoeste do Rio Grande do Sul, envolvendo 18 municípios, foram encontrados 48,7% de animais positivos (HERRMANN et al., 2004). Em amostras sorológicas de 587 cães procedentes de 6 municípios da região sul do Rio Grande do Sul, foi encontrada uma prevalência de 25,38%, distribuída em 38,92% para *canicola*, 28,85% para *grippotyphosa*, 8,05% para *pyrogenes*, 7,38% para *copenhageni*, 4,02% para *icterohaemorrhagiae*, e outros em menor frequência (MACHADO et al., 1999). No município de Pelotas, a avaliação da prevalência e fatores de risco para a leptospirose canina identificou a

prevalência de 28,9% em cães domiciliados, sendo predominantes reações para os sorovares *canicola* e *icterohaemorrhagiae* (FURTADO et al., 1997). No município de Santa Cruz do Sul verificou-se a ocorrência de 36,4% de cães soropositivos em 2002, aumentando para 56% em 2003, e os principais sorovares encontrados nesta espécie foram o *grippotyphosa*, *australis*, *icterohaemorrhagiae*, *autumnalis*, *pomona* e *bratislava*. Neste mesmo município houve 24% e 51,2% de bovinos positivos nos anos de 2003 e 2004, respectivamente, e de 40 e 55,5% no caso dos suínos (LOBO et al., 2004). Entre 1427 amostras de soro de cães suspeitos examinados no período compreendido entre os anos 2000 e 2002, no Centro de Pesquisa Veterinária Desidério Finamor (CPDVF-FEPAGRO), no Rio Grande do Sul, 754 (52,83%) foram positivos para leptospirose. O maior número de positivos ocorreu para o sorovar *copenhageni*, seguido de *canicola* e *bratislava*, havendo reações para títulos mais baixos para *tarassovi*, *icterohaemorrhagiae*, *pyrogenes* e *australis* (OLIVEIRA & PIRES NETO, 2004). Em Porto Alegre, foram avaliados 86 cães com suspeita clínica de leptospirose, e 89 clinicamente sadios, atendidos no HCV-UFRGS. Os dois grupos apresentaram animais com altos títulos sorológicos (ambos 37%), não diferindo entre si. Os principais sorovares encontrados foram *icterohaemorrhagiae*, *copenhageni*, *canicola*, *wolffi*, *castellonis*, *australis*, *pyrogenes* e *bratislava* (SANTIN et al., 2006). Em outro trabalho realizado nesta mesma instituição, entre janeiro de 2005 e julho de 2007, 67% dos cães suspeitos de leptospirose foram positivos, sendo o sorovar *icterohaemorrhagiae* o mais prevalente, seguido do *copenhageni* (DALMOLIN & GONZÁLEZ, 2007).

3.6. Fases e sinais clínicos da leptospirose

A apresentação clínica da leptospirose é bifásica, com a bacteremia durando cerca de uma semana, seguida da fase imune, caracterizada por produção de anticorpos e excreção de leptospiras na urina (LEVETT, 2001). A excreção urinária das bactérias passa a ser intermitente, podendo persistir por períodos de tempo de longa duração, variáveis com a espécie animal e o sorovar envolvido (VASCONCELLOS, 1993). Os principais fatores na patogênese das lesões renais são relacionados à presença das leptospiras por sua migração e produção de toxinas. As alterações túbulo-intersticiais podem ser reversíveis se o tratamento for iniciado precocemente. No entanto, se não tratada, a doença pode levar ao estado de portador renal crônico, no qual as

leptospiras se instalam e permanecem viáveis nos túbulos renais, apesar da presença da imunidade humoral ou celular do hospedeiro (YANG et al., 2001).

Os sinais clínicos mais comuns na infecção aguda em cães são: letargia, depressão, anorexia, vômito, febre, poliúria, dor abdominal, diarreia, icterícia, petéquias e mialgia (SHERDING, 1998). Os sinais gastrintestinais tendem a ser mais severos e persistentes em cães com leptospirose, se comparados com outras causas de insuficiência renal aguda (LANGSTON & HEUTER, 2003). As manifestações clínicas apresentam variações, dependendo da susceptibilidade de cada indivíduo e do sorovar infectante (GREENLEE et al., 2005). Os animais que sobreviverem podem tornar-se insuficientes renais crônicos. O cão é considerado o hospedeiro definitivo do sorovar canicola, ou seja, seria adaptado a este sorovar e teoricamente não deveria apresentar a doença clínica, porém o que se observa é que o cão infectado com o sorovar também pode apresentar sinais clínicos. A infecção por este sorovar resulta no comprometimento renal, que pode se manifestar sob a forma de uremia e sinais gastrentéricos (VAN DE MAELE et al., 2008).

Alguns cães que sobrevivem à infecção aguda desenvolvem nefrite intersticial ou hepatite ativa crônica. Poliúria, polidipsia, perda de peso, ascite e sinais de encefalopatia devido à insuficiência hepática são as manifestações mais comuns da leptospirose crônica (LANGSTON & HEUTER, 2003; LAPPIN, 2003).

3.7. Diagnóstico laboratorial

O diagnóstico definitivo da leptospirose baseia-se na detecção de anticorpos séricos ou na detecção de leptospiras no material clínico, como urina, sangue e líquido (VAN DE MAELE et al., 2008).

Sorologia: Anticorpos são detectáveis no sangue aproximadamente cinco a sete dias após o início dos sinais clínicos (LEVETT, 2001) e geralmente atingem os níveis mais altos dentro de três a quatro semanas. Os níveis de anticorpos então gradualmente diminuem, mas podem permanecer detectáveis por anos (AHMAD et al., 2005).

O teste de aglutinação microscópica (MAT) permanece sendo a investigação sorológica definitiva. A gama de antígenos usada deve incluir sorovares representativos de todos os sorogrupos e sorovares comuns na região (sorovares locais). O MAT não pode diferenciar

aglutinação de anticorpos devido à infecção atual, recente ou passada. Idealmente, assim como outros testes sorológicos, duas amostras de soro consecutivas devem ser examinadas quanto à soroconversão de quatro vezes ou mais de aumento no título. A maior vantagem do MAT é sua alta especificidade, porém os pacientes com leptospirose podem produzir anticorpos que reagem com vários sorovares. Este fenômeno, chamado reação-cruzada, é frequentemente observado na fase inicial da doença (WORLD HEALTH ORGANIZATION, 2003). Quando a doença se torna crônica, o título de anticorpos pode ficar reduzido e por isto muitos animais são portadores soronegativos. Após a infecção, as leptospiras localizam-se nos rins e são excretadas de forma intermitente na urina (LEVETT, 2001). Cães portadores do sorovar canicola podem secretar ativamente leptospiras na urina, com títulos sorológicos menores que 1:100 (LANGSTON & HEUTER, 2003).

Exame direto: é realizado através de microscopia de campo escuro, na urina ou no soro. São necessários aproximadamente 10^5 organismos/mL para a visualização e, além disso, uma variedade de bactérias poderia ser confundida com leptospiras. Devido a estas considerações, este exame não é recomendado como diagnóstico isolado (GREENE et al., 2006).

Cultura: a cultura do sangue deve ser feita assim que possível depois da apresentação do paciente; a da urina pode ser feita nos casos em que se suspeite do estado de portador. Apesar da cultura confirmar o diagnóstico, é raramente utilizada, por ser complicada, cara, consumir tempo, ser tecnicamente exigente, requerer incubação prolongada e ter baixa sensibilidade (AHMAD et al., 2005).

Diagnóstico molecular: A reação em cadeia da polimerase (PCR) é um método *in vitro* para amplificar seletivamente uma sequência de DNA alvo-específica (MÉRIEN et al., 1992). Vários pares de oligonucleotídeos iniciadores para detecção das leptospiras patogênicas por PCR foram descritos (GRAVEKAMP et al., 1993; LEVETT, 2001; HARKIN et al., 2003). A PCR é um método mais sensível no diagnóstico precoce e crônico da leptospirose quando comparada ao MAT, uma vez que a sorologia negativa não descarta a fase hiperaguda ou a fase leptospirúrica da doença (CHARELLO et al., 2006). Uma limitação do diagnóstico da leptospirose baseado em PCR é a incapacidade da maioria dos testes em identificar o sorovar infectante. Apesar de isto não ser importante para o manejo individual do paciente, a identificação do sorovar tem valor significativo epidemiológico e de saúde pública (LEVETT, 2001; AHMAD et al. 2005).

Técnicas moleculares para a identificação genotípica do sorovar são descritas, como as baseadas em análise de variação do número de repetições em tandem (análise de VNTR, do inglês variable-number tandem-repeat) (MAJED et al., 2005; SALAUN et al., 2006).

3.8. Patologia clínica

As alterações encontradas no hemograma, bioquímica sérica e urinálise não são exclusivas da leptospirose, porém auxiliam o clínico a avaliar o estado do paciente e definir o melhor tratamento. Apesar de algumas alterações serem consideradas “clássicas” na leptospirose, é importante lembrar que resultados normais não excluem a doença.

Hematologia: as alterações hematológicas observadas nos casos de leptospirose usualmente são leucocitose por neutrofilia e graus variáveis de anemia. A leucopenia pode ser um achado na fase inicial da infecção (leptospiremia), evoluindo geralmente para leucocitose com desvio a esquerda, com a progressão da doença (LANGSTON et al., 2003; GREENE et al., 2006).

Bioquímica sérica: as dosagens de uréia, creatinina, alanina aminotransferase (ALT), fosfatase alcalina (FA) e bilirrubina constituem-se nos principais exames de monitoramento da evolução do quadro clínico e, conseqüentemente, do prognóstico de animais com leptospirose (VAN DE MAELE et al., 2008). Exames de função renal na leptospirose canina revelam frequentemente aumento dos níveis séricos de uréia e creatinina, variando segundo o grau de comprometimento renal (GREENE et al., 2006). A função renal nos cães que sobrevivem à infecção subaguda pode retornar ao normal dentro de duas a três semanas ou pode evoluir para insuficiência renal crônica poliúrica compensada. As alterações das enzimas hepáticas ALT e FA, assim como os níveis séricos de bilirrubina, variam com a severidade da lesão hepática (LANGSTON & HEUTER, 2003). Outras alterações bioquímicas que podem ocorrer devido à doença renal, hepática, perdas gastrintestinais ou acidose são: hipocalemia, hiperfosfatemia, hipoalbuminemia, hiponatremia, hipocalcemia e azotemia (GONZÁLEZ & SILVA, 2006). Hiperglobulinemia é detectada em alguns cães com leptospirose crônica (LAPPIN, 2003). A creatina quinase (CK) aumenta com a inflamação muscular (LANGSTON & HEUTER, 2003; WOLFFENBÜTTEL et al., 2004). Em alguns casos com anúria terminal, hipercalemia está presente (LANGSTON & HEUTER, 2003).

A proteína C-reativa sérica, uma proteína de fase aguda positiva, foi investigada em cães com leptospirose experimentalmente induzida (CASPI et al., 1987) e naturalmente adquirida (YAMAMOTO et al., 1993), apresentando aumento em sua concentração. As proteínas de fase aguda são consideradas sensíveis, porém pouco específicas, já que suas alterações ocorrem em processos infecciosos, inflamatórios, neoplásicos ou traumáticos. A utilidade destas proteínas se baseia no fato de indicar estes processos, mesmo se estes forem subclínicos (CERÓN et al., 2005).

Urinálise: a urinálise e a determinação das concentrações séricas de creatinina e uréia são métodos convencionais utilizados para a avaliação da função renal. (BARTGES, 2004). O envolvimento renal pode variar de curso subclínico, com proteinúria branda e alterações leves no sedimento urinário, até insuficiência renal grave. A severidade da doença é amplamente determinada pela virulência das leptospiras, a carga bacteriana e a defesa do hospedeiro. Durante a fase septicêmica, eritrócitos, leucócitos e cilindros granulares estão geralmente presentes (VISITH & KEARKIAT, 2005). Na urinálise de cães com leptospirose observam-se geralmente densidade baixa, glicosúria, proteinúria e bilirrubinúria (que normalmente precede a hiperbilirrubinemia), acompanhadas de elevação de cilindros granulosos, leucócitos e eritrócitos no sedimento urinário (LANGSTON & HEUTER, 2003). Na insuficiência renal aguda, pode ocorrer glicosúria normoglicêmica devido à presença de lesões tubulares significativas. Cilindros são detectados em cerca de 30% dos cães com insuficiência renal aguda, mas sua ausência não exclui o diagnóstico de lesão parenquimatosa aguda (COWGILL & ELLIOT, 2004).

3.9. Tratamento

O tratamento da leptospirose em cães é baseado na reposição do equilíbrio hidroeletrólítico, energético e no uso de antimicrobianos. Nos casos severos de anemia ou coagulação intravascular disseminada (CID) faz-se necessária a realização de transfusão sanguínea (SESSIONS & GREENE, 2004). A diurese com agentes osmóticos ou diuréticos tubulares é necessária para os animais oligúricos (GREENE et al., 2006). A hemodiálise pode aumentar a chance de sobrevivência em cães com insuficiência renal oligúrica ou anúrica (ADIN & COWGILL, 2000; LAPPIN, 2003).

O papel dos antimicrobianos na leptospirose inclui terapia para infecção aguda e prevenção de doença clínica com profilaxia semanal, sendo a profilaxia recomendada apenas para humanos, evitando a seleção da resistência aos antimicrobianos (GREENE et al., 2006). O pequeno número de agentes antimicrobianos que provaram ser efetivos, através de estudos controlados, tem aplicação limitada em certas populações de pacientes devido à toxicidade, contra-indicação, custo ou administração complexa (MOON et al., 2007). Uma grande dificuldade em avaliar a eficácia do tratamento com antimicrobiano resulta na apresentação tardia de vários pacientes com doença grave, após as leptospiras já terem se localizado nos tecidos (LEVETT, 2001). A terapia antimicrobiana é direcionada inicialmente para resolver a fase leptospirêmica e, subsequentemente, a fase leptospirúrica (portador renal) (LANGSTON & HEUTER, 2003).

A *Leptospira* é suscetível a uma ampla gama de agentes antimicrobianos *in vitro*. No entanto, estudos indicam que a suscetibilidade *in vitro* não se correlaciona com a eficácia dos antimicrobianos *in vivo*. A diferença dos resultados *in vitro* e *in vivo* pode ser atribuída a concentrações inefetivas da droga no rim, no foco de persistência das leptospiras. A comparação direta entre estudos é difícil devido ao uso de diferentes sorovares, diferentes preparações de fármacos e diferentes rotas de administração (ALT & BOLIN, 1996).

A terapia dos animais domésticos acometidos pela leptospirose objetiva não somente o restabelecimento do doente, mas, particularmente, a eliminação da infecção renal que propicia a contaminação do ambiente (GUIMARÃES et al., 1982/83). Os fármacos mais sugeridos para eliminar as leptospiras dos rins, em diferentes espécies (hamster, bovinos, cães) são a estreptomicina (ALT & BOLIN, 1996; GIRIO et al., 2005), dihidroestreptomicina (GERRITSEN et al., 1994; SHERDING, 1998) e doxiciclina (TRUCCOLO et al., 2002; LANGSTON & HEUTER, 2003; BURR et al., 2009).

3.10. Imunização

A imunização é sorovar-específica e protege apenas contra a doença causada pelo sorovar homólogo ou sorovares similares antigenicamente. Portanto, as vacinas precisam conter sorovares representativos daqueles presentes na população que será imunizada (LEVETT, 2001).

As vacinas caninas geralmente contêm antígenos que imunizam contra os sorovares icterohaemorrhagiae e canicola, mas cães imunizados podem ser infectados com sorovares que não estejam contidos nas vacinas comerciais (GREENE et al., 2006). Visto o aparecimento cada vez maior de outros sorovares em cães, isto reforça ainda mais a importância da pesquisa continuada no desenvolvimento de novas vacinas contra a leptospirose e a necessidade da inclusão de novos sorovares, visando à elaboração de vacinas mais efetivas e com imunidade mais duradoura (BATISTA et al., 2005).

4. RESULTADOS E DISCUSSÃO

Os resultados e discussão deste trabalho são apresentados na forma de artigos científicos. Cada artigo foi formatado de acordo com as orientações das revistas científicas às quais foram submetidos e cada subtítulo deste capítulo corresponde a um dos artigos. Os comprovantes de submissão ou aceite encontram-se em anexo.

ARTIGO 1

Clinical and laboratorial findings of leptospirosis in naturally infected dogs of Southern Brazil.

Submetido para publicação na Veterinary Microbiology (comprovante em ANEXO 1).

ARTIGO 2

Diagnosis and doxycycline treatment follow-up of leptospirosis in four naturally infected dogs.

Será submetido para publicação na Veterinary Record

ARTIGO 3

Serum and urinary C-reactive protein concentrations in dogs with leptospirosis.

Aceito para publicação na Acta Scientiae Veterinariae (comprovante em ANEXO 2).

4.1. ARTIGO 1

Clinical and laboratorial findings of leptospirosis in naturally infected dogs of Southern Brazil

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Abstract

Leptospirosis is a zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. Manifestations of disease in dogs vary from asymptomatic carriers to severe clinical signs and death. The objective of this study was to characterize *Leptospira* infections in three different dog

populations in Porto Alegre, Southern Brazil: dogs suspected of having leptospirosis (Veterinary Hospital dogs), dogs from Control Center of Zoonoses (shelter dogs) and dogs from Arquipélago (a neighborhood with a high prevalence of human leptospirosis). The diagnosis was based on serology and PCR in blood and urine. A total of 14.6% (37/253) and 14.2 % (36/253) of all dogs tested positive by PCR in blood and urine, respectively, and 48.2% (122/253) had a positive serology. The most prevalent serovars were canicola, icterohaemorrhagiae and copenhageni. The species *Leptospira kirschneri* was found only in dogs from Arquipélago. The presence of rats in the environment was associated with positive PCR in urine (P=0.02). Although increased serum creatinine (P=0.009), icterus (P=0.004) and glucosuria (P=0.04) were associated with leptospiruria in the Veterinary Hospital group, the absence of clinical signs or alterations in Complete Blood Count (CBC), serum biochemistry or urinalysis did not exclude the infection.

Keywords: leptospirosis, dog, prevalence, diagnosis.

Introduction

Leptospirosis is a worldwide distributed zoonotic disease. Although infection may lead to clinical disease, subclinical infections in animals and humans may occur. Contaminated urine from maintenance and incidental hosts is reportedly the main source for dissemination of the pathogenic leptospire. Rodents play an important role as maintenance hosts, but dogs can be significant reservoirs for human infection in tropical areas as well as the source of disease outbreaks in tropical areas (Levett, 2001). In Rio Grande do Sul State, Southern Brazil, a high incidence of leptospirosis in humans has been reported; there are about 10 cases/100,000 inhabitants, which is greater than the country's average (3.5cases/100,000 inhabitants) (Barcellos

et al., 2003). Porto Alegre, the most populous City in this State, reported 0.85 to 7.14 cases/100,000 inhabitants between 1996 and 2007 (Thiesen et al., 2008).

Clinical signs of canine leptospirosis depend on the age and immunological status of the host, environmental factors, and the virulence of the infecting serovars (Langston and Heuter, 2003; Ko et al., 2009). Although non-specific and variable, clinical signs may include anorexia, lethargy, fever, vomiting, icterus, polydipsia and polyuria (Burr et al., 2009; Guerra, 2009). Some dogs develop a severe form of the disease with overt signs, but most dogs present just a subclinical infection (Van de Maele et al., 2008). In the past, the most common serovars related to cases of canine leptospirosis worldwide were canicola and icterohaemorrhagiae. After the introduction of a vaccine, an apparent decrease in the prevalence of these serovars occurred in some countries, as the observed the United States and Canada where a shift in the predominant serovars that causes clinical signs in dogs (grippityphosa, pomona, bratislava, and more recently, autumnalis) were reported (Langston and Heuter, 2003; Ward et al., 2004; Goldstein et al., 2006). In Brazil, copenhageni and icterohaemorrhagiae are the serovars most prevalent. These data may not reflect all of the serovars infecting the canine population as atypical and/or subclinical infections are likely to be underestimated. In addition, not all serovars are included in the routine serological test panel (Burr et al., 2009).

Organs system involvement in leptospirosis is thought to be somewhat serovar specific. Thus, abnormalities detected on hematology and chemistry panels depend on the organs involved as well as the stage of the disease. Leukocytosis, neutrophilia, and thrombocytopenia are commonly reported hematologic findings; however neutropenia or normal white cell count may also occur. On biochemistry profile, azotemia with or without increases in hepatic enzymes and bilirubin are the most common abnormal findings. Urinalysis may reveal hematuria, proteinuria,

bilirubinuria, isosthenuria or hyposthenuria, and, occasionally, glucosuria due to tubular damage (André-Fontaine, 2006; Burr et al., 2009).

Diagnosis of leptospirosis is based on antibody response and/or detection of the organism associated with typical clinical signs (Burr et al., 2009). Although the detection of antibodies by the microscopic agglutination test (MAT) has been historically applied for diagnosis of leptospirosis and is a useful tool for epidemiological studies, this technique is not fully reliable. Following infection, it may take 7-10 days before an antibody response can be detected; cross-reaction between serovars is known to occur, and vaccination may cause a serologic response that is mistaken for infection (Levett, 2001; Burr et al., 2009). Application of the polymerase chain reaction (PCR) assay for detection of leptospires in blood and urine of dogs has been previously described (Harkin et al., 2003a; Branger et al., 2005; Khorami et al., 2009). When comparing PCR assays, the best combination of sensitivity and specificity was achieved using an assay developed by Gravekamp et al. (1993). This assay detects seven species of pathogenic leptospires, including *L. kirschneri* that is missed by many of the other PCR assay (Gravekamp et al., 1993; Van de Maele et al., 2008). PCR is especially useful for early diagnosis of leptospirosis and to detect chronic carriers (Harkin et al., 2003b; Van de Maele et al., 2008).

The aim of this study was to characterize *Leptospira* infections in 3 different dog populations in Porto Alegre, Southern Brazil including: 1) a zoonotic disease control center; 2) an isolated, neighborhood with endemic human infection; and 3) a referral veterinary teaching hospital. An additional goal was to identify any differences in the clinical signs, risk factors and clinicopathologic aspects of the disease produced by different suspected infecting leptospire on the basis of serology (MAT) and PCR results.

Materials and Methods

Dog populations

Between May 2007 and February 2009, 253 dogs from Porto Alegre, Southern Brazil, were enrolled in the study. Three populations were evaluated including dogs from Arquipélago, dogs from Control Center of Zoonosis (CCZ) and dogs presented to the Veterinary Hospital of Federal University of Rio Grande do Sul (Table 1). Animals receiving antibiotic therapy at the time of evaluation were excluded from the study. Owners have agreed to volunteer participate in this study.

Dogs from Control Center of Zoonoses (CCZ): CCZ is a shelter and control center for zoonotic diseases supported by the county health secretary that rescues stray dogs and receives relinquished dogs. Stray dogs from the city streets are impounded by CCZ; if healthy and unclaimed, the dog is offered for adoption.

Dogs from Arquipélago neighborhood. This neighborhood is a flood area, without sanitation, and having a high population of dogs and rodents; Arquipélago neighborhoods were previously reported as endemic for human leptospirosis (Henkes, 2008). The inhabitants rely on garbage recycling they collect from nearby Porto Alegre city for living, and as consequence the islands are being used as a garbage dump. We investigated two of the larger, habited islands, Ilha Grande dos Marinheiros and Ilha do Pavão. Samples were collected from dogs \geq 1 year-old, independent of the dog's health condition. Owners were asked to answer a questionnaire and blood and urine samples were obtained from their dog(s) after clinical examination.

Dogs referred to Veterinary Hospital. Dogs suspected of acute or chronic leptospirosis were selected. The criteria for inclusion were 1) the presence of at least one risk factor (rats in the environment or unvaccinated dog living outdoor) and 2) two or more clinical signs (icterus,

anorexia/weight loss, vomiting, diarrhea, polyuria/polydispsia, fever) or laboratorial alterations such as leukocytosis, high serum creatinine or high alanine aminotransferase (ALT).

Questionnaire

Questionnaire applied to owners included information such as breed gender, age, vaccination status (not vaccinated, vaccinated <1 year ago, vaccinated >1 year ago), environment, contact with other animals, presence of rodents in the household, clinical signs, medications and if owners had leptospirosis diagnosed in the previous two years. Animals currently receiving antibiotic therapy were not included in the study.

Sampling

Urine: Urine was collected by voiding or catheterization. In order to neutralize the urine for DNA extraction, PBS (pH 7.4) in a proportion of 1:2.5 v/v was added to the sample immediately after collection. Another aliquot was kept without PBS for subsequent urinalysis (performed within 4 hours). The PBS aliquot was subjected to DNA extraction, which was completed within 8 hours of sample collection. Both samples were refrigerated (4°C) until processing.

Blood: Samples were collected from cephalic, lateral saphenous or jugular vein in vacuum tubes with no additive and EDTA tubes. Tubes with no additive were centrifuged at 600×g for 15 minutes, and the serum was stored at -20°C for serological and biochemical analysis. For the Complete Blood Count (CBC), EDTA samples were refrigerated up to 6 hours after collection. An aliquot of EDTA blood sample was frozen at -20°C until DNA extraction.

DNA extraction

DNA was extracted from EDTA-blood samples using a commercial kit following manufacturer's protocol (QIAamp DNA Blood Mini kit, QIAGEN, Valencia, CA, USA). An

internal control target, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was tested in all DNA samples from blood to insure successful extraction, as previously described (Santos et al., 2009).

DNA extraction from urine samples was performed in duplicate, using a modified protocol (Lucchesi et al., 2004). Briefly, a total of 1.5 mL of the mixture (urine + PBS pH 7.4) was incubated at 37°C for 10 minutes, and then centrifuged at 800×g at room temperature. The supernatant was transferred to another tube and centrifuged at 1560×g for 20 minutes. After the supernatant was discarded, the pellet was resuspended and washed with 1 mL of PBS, and then centrifuged at 1560×g for an additional 20 minutes. The supernatant was discarded and the pellet was resuspended in 100 µL of PBS and boiled for 10 minutes. DNA was stored at -20°C until the molecular analysis. The efficacy of this procedure for obtaining DNA was verified using various concentrations of *Leptospira* spiked samples of urine.

Samples analysis and interpretation of results

Serology: Microscopic agglutination test (MAT) (World Health Organization, 2003) was performed to detect the presence of antibodies against 13 *Leptospira* antigens, considering titers $\geq 1:100$ as positives, and highest titer when more than one serovar reacted. The tested serovars included australis, autumnalis, bratislava, canicola, copenhageni, grippityphosa, hardjo, hebdomadis, icterohaemorrhagiae, pomona, pyrogenes, tarassovi and wolffi.

Polymerase chain reaction (PCR): For detection of pathogenic leptospires in blood (leptospiemia) and urine (leptospiuria) the primer sets G1/G2 and B64-I/B64II (Gravekamp et al., 1993) were used to amplify a fragment of DNA from *secY* and *flaB* genes, respectively. G1 (5' CTG AAT CGC TGT ATA AAA GT 3') and G2 (5' GGA AAA CAA ATG GTC GGA AG 3') amplify DNA of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchi*, *L. santarosai*, and *L.*

meyeri, whereas B64-I (5' ACT AAC TGA GAA ACT TCT AC 3') and B64-II (5' TCC TTA AGT CGA ACC TAT GA 3') amplify DNA of *L. kirschneri*. They generate a 285-bp and 563-bp, respectively.

PCR was performed in a reaction containing 1× buffer, 1.5 mM MgCl₂, 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 5 pmol of each primer, 1.25 U of *Taq* DNA polymerase (GoTaqFlexi DNA Polymerase, Promega, Madison, WI, USA), 5 μL of the DNA template and ultrapure water up to 25 μL. A positive control (DNA extracted from leptospire's cultures) and two negatives controls (ultrapure water) were included in each run. An initial step at 95°C for 2 minutes was followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds; the final extension was done at 72°C for 5 minutes into a thermal cycler (iCycler Biorad, Hercules, CA, USA or Eppendorf Mastercycler Gradient, Foster City, CA, USA). After electrophoresis (1 h at 100 V) of 15 μL of the reaction solution in a 1.5% agarose gel with 0.1g/mL ethidium bromide, visualization and photography of the bands of the expected size products were performed under UV light (Epi Chemi II Darkroom, UVP Inc., Upland, CA, USA) using the VisionWorks LS Analysis Software (Upland, CA, USA).

Fragments of 285 bp and 563 bp, amplified from *L. interrogans* serovar canicola strain Hond Utrecht IV and *L. kirschneri* serovar grippotyphosa strain Moskva V, respectively, were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Gaithersburg, MD, USA) and serial dilutions were tested to define the detection limit of the PCR. Since the singleplex PCR assays showed better sensitivity than previously reported duplex assay, these were used for subsequent sample analysis (data not shown).

Hematology: Red Blood Cells (RBC), White Blood Cells (WBC) and hemoglobin determinations were performed using a semiautomatic cell counter (CC 530 CELM, Brazil) and the hematocrit was obtained by the microhematocrit centrifugation method. Specific leukocyte differentials were performed on blood smear by light microscopy.

Serum biochemistry: Creatinine and ALT were measured by spectrophotometry in a semi-automatic analyzer (Metrolab 1600, Buenos Aires, Argentina) using commercial kits (Labtest, Lagoa Santa, MG, Brazil).

Urinalysis: Specific gravity was measured by refractometry, urine pH, protein, glucose, bilirubin and ketones were measured using a urinary dipstick, and sediment examination was performed by light microscopy.

Statistical analysis:

Association between the parameters including blood analysis, urine analysis, clinical signs and each of blood PCR, urine PCR, and serological results was done using Chi square test, or Fisher's exact when appropriate using Stata 11 (StataCorp, College Atation, TX 77845, USA). Continuous variables such as blood cells count, serum enzyme concentration, and urinalysis parameters were transformed into categorical variables (Table 2). Multivariable logistic regression model with forward stepwise variable selection was used to evaluate the association between each of the outcomes (blood PCR, urine PCR, and serology) and blood and urine analysis parameter, clinical signs, as well as the environmental risk factors. Goodness of fit test was used to assess the model fitness and variable selection. A p-value ≤ 0.05 was considered significant.

Results

Internal control and sensitivity of PCR

The internal control, GAPDH, was positive for all blood samples, confirming adequate DNA extraction. The sensitivity testing of the singleplex PCR showed that leptospire were consistently detected (100%) at 10 copies/reaction for primers G1/G2 and 50 copies/reaction for primers B64-I/B64-II; 5 and 25 copies/reaction for primers G1/G2 and B64-I/B64-II could be detected, but less consistently with a success rate of 5 /10 and 4/10 positive PCR reactions per attempts, respectively (data not shown).

Diagnosis of leptospirosis according to PCR and serology

A total of 14.6% (37/253) and 14.2 % (36/253) of all dogs tested positive by PCR in blood and urine, respectively (Table 3). Of these, 2.0% (5/253) of the dogs had concurrent leptospiremia and leptospiruria. The overall prevalence of leptospiremia among dogs from the CCZ (7.7%) was statistically similar to dogs from the Arquipelago neighborhood (14.8%), whereas the Veterinary Hospital group had the highest prevalence (27.3%), which was statistically different from the CCZ group. The prevalence of leptospirosis based on positive PCR in urine and positive serology was the lowest among dogs from Arquipelago, which was statistical different when compare with the other groups. Most of the positive PCR results were detected using primers G1/G2. Using primers B64-I/B64II, 6 blood and 2 urine samples from Arquipelago were positive, demonstrating the presence of the species *L. kirschneri* in this population only. Of these dogs, 4/6 dogs with leptospiremia had some clinical sign, including vomiting and diarrhea, anorexia and weight loss, and polyuria/polydipsia, whereas the two dogs with leptospiruria were clinically unaffected.

There was no association between seropositivity and positive PCR in blood or urine ($P>0.05$). A total of 48.2% (122/253) dogs reacted with one or more serovars, but only 34.4% (42/122) of seropositive dogs had positive PCR in blood and/or urine. All 13 serovars tested on MAT reacted at least once. When the identification of the infecting serovar was possible (higher titer or reactive to one serovar only), canicola, icterohaemorrhagiae and copenhageni were the most prevalent (57/122). Titers $\geq 1:200$ (1:200 to 1:1,600) were detected in four dogs only, including two against serovar canicola and two against serovar copenhageni. Three of these dogs tested negative by PCR in blood and urine, however the dog having a titer 1:1,600 (serovar copenhageni) was positive by PCR (G1/G2 primers) in urine.

Risk factors

With the exception of breed and gender, which were evaluated in all 3 groups, risk factors identified by questionnaire responses were only analyzed in Arquipelago and Veterinary Hospital populations. There were no associations between positive PCR or positive serology and breed, gender or age. The presence of rats in the environment was associated with positive PCR in urine ($P=0.02$), but not with positive PCR in blood or with positive serology. All dogs from Arquipelago had direct or indirect contact with rats; i.e., presence of these rodents in the household, and indeed some of them were rat hunters. Five owners' dogs from Arquipelago had a previous diagnosis of leptospirosis, but their dogs had no leptospiruria at the time this study was carried out. Nevertheless, two of these dogs had leptospiremia. The absence of vaccination against leptospirosis was associated with the presence of positive PCR in urine when considering the groups together ($P=0.0001$), but not when analyzing the two groups individually. There was no association between vaccination and positive PCR in blood.

Only 6 dogs were vaccinated less than one year before presenting to the Veterinary Hospital; one of these dogs had leptospiremia, three had leptospiruria and one had concurrent leptospiremia and leptospiruria. There was a significant association between serology and vaccination status in Veterinary Hospital group ($P=0.047$), but not in Arquipélago. Contact with other animals was not associated to leptospirosis in the studied populations.

Clinical signs

Association between positive urine PCR and icterus was observed in Veterinary Hospital ($P=0.004$) group, but not between positive blood PCR and icterus. Although 24.6% (16/65; two of these dogs having concurrent positive PCR in blood and urine) of the dogs from CCZ and 22.6% (35/155, with one dog having concurrent positive PCR in blood and urine) from Arquipélago showed positive PCR in blood and/or urine, none of them were icteric. Multivariable logistic regression showed that icteric dogs had 21 times the odds of being PCR urine positive comparing to non-icteric dogs. An association between bad body condition and positive PCR in urine was found in Veterinary Hospital patients ($P=0.038$) but not in Arquipélago dogs. Other clinical signs of dogs from Arquipélago and Veterinary Hospital, including anorexia/weight loss, vomiting, diarrhea and polyuria/polydispsia, were not significantly associated with the leptospiremia or leptospiruria. An association between diarrhea and positive serology ($P=0.044$) was found. Dogs with diarrhea had 4.67 times the odds of being PCR blood positive compared to dogs having no diarrhea. Rectal temperature was performed in the Veterinary Hospital population only, but a positive association was not observed between fever or hypothermia and positive serology or positive PCR in blood or urine.

Clinicopathologic aspects

Statistically significant differences were found among groups when considering anemia ($P=0.012$); 34.1% and 36.3% of the dogs from the Arquipélago and Veterinary Hospital groups had anemia, respectively. This was statistically different from the CCZ group, where anemia was found only in 16.9% of the dogs. However, no association between anemia and positive PCR or serology was observed. There was no association between positive serology, PCR in blood or urine and other CBC alterations (total and differential leukocyte count, red blood cells parameters). Although serum ALT ($P=0.001$) and creatinine ($P=0.0001$) were significantly different among population groups, high serum ALT was not associated with positive PCR or serology results. On the other hand, high values of serum creatinine were associated with positive urine PCR ($P=0.0091$) and with positive serology ($P=0.0002$), but not with positive blood PCR results.

A significant association was also observed between positive PCR in urine and glucosuria ($P=0.002$), but not between PCR in blood and glucosuria. When individually observed, only the Veterinary Hospital group had a significant association between positive PCR in urine and glucosuria ($P=0.04$). Proteinuria was associated with positive PCR in the Arquipélago group ($P=0.026$). There was a difference between positive serology and leukocyturia ($P=0.013$) in the Veterinary Hospital group. Significant associations were not demonstrated between other urinary parameters (pH, ketones, bilirubin, blood, specific gravity, and sediment) and positive PCR in blood or urine. However, there was a difference between the population groups in urinary pH ($P<0.0001$) with aciduria more common in the Veterinary Hospital group.

Discussion

In this study, the occurrence of *Leptospira* infections was evaluated in 3 different dog populations. While positive PCR for leptospires in blood was interpreted as indicating an acute stage of disease, a positive PCR in urine and/or serology was taken to indicate a chronic stage of infection and production of antibodies (Levett, 2001). Dogs that had concurrent leptospiremia and leptospiruria were probably in the transition from acute to chronic stage of the disease. The highest number of dogs diagnosed with leptospirosis based on PCR of blood and urine as well as serology was observed in the Veterinary Hospital population. This finding was expected since these dogs had risk factors and clinical signs or laboratory abnormalities that were suspicious for leptospirosis (Langston and Heuter, 2003; Burr et al., 2009). In CCZ dogs, the percentage of dogs with leptospiruria was higher than leptospiremia. Although this may reflect the leptospirosis picture of stray dogs in the city, it is also possible for the infection to have been acquired at CCZ as multiple dogs were housed in each pen. It was somewhat surprising that the prevalence of leptospiremia was higher than the prevalence of leptospiruria in the Arquipélago dogs. However, there are several plausible explanations for this finding. A recent outbreak of leptospirosis on the island is one possibility, however the authors also speculate that different serovars may infect dogs on the island that are less likely to localize to the kidney or perhaps these dogs are genetically resistant to certain serovars. The lower number of seropositive dogs on Arquipélago in comparison to the CCZ and Veterinary Hospital populations also may be explained by the presence of different serovars on the island, which were not detected by the routine serologic testing (Burr et al., 2009).

The higher percentage of dogs with positive serology in this study compared to positive PCR suggests that dogs develop immunity against some serovars and do not become carriers.

The most common serovars identified were *canicola*, *icterohaemorrhagiae* and *copenhageni*, which were consistent with the findings of other studies involving dogs in Rio Grande do Sul state (Machado et al., 1999; Oliveira and Pires Neto, 2004; Santin et al., 2006). Titers for *copenhageni* and *icterohaemorrhagiae* were often concurrently positive by MAT, likely related to their serologic cross-reactivity (Zwijnenberg et al., 2008). Other studies documented that *grippotyphosa* (Machado et al., 1999; Lobo et al., 2004) and *bratislava* (Oliveira and Pires Neto, 2004) were also common serovars in dogs of Brazil, however these serovars were infrequently recognized in the study herein. Since most vaccines in Brazil protect against *icterohaemorrhagiae* and *canicola*, the presence of titers against these serovars could be related to vaccination and must be evaluated with caution (Guerra, 2009). It is also possible that these titers were due to natural infections, since most of the dogs had not received a vaccination in the past year.

Serology has been reportedly shown as a poor predictor of urinary shedding of leptospire in dogs (Harkin et al., 2003b). In our study, there were 26/253 (10.3%) dogs that had leptospiremia and/or leptospiruria that tested negative on MAT. Negative serology in the presence of positive PCR could be attributed to an early stage of the disease (Ooteman et al., 2006), a serovar not included in the panel of tested MAT (Brod et al., 2005), or to lower titers (less than lowest MAT titer) of antibodies in the chronic stage; thus, these animals are seronegative carriers (Levett, 2001). Dogs are well adapted to serovar *canicola* and may actively shed leptospire with titers under 1:100 (Langston and Heuter, 2003).

The species *L. kirschneri* was found only in dogs from the Arquipélago. Although the serogroup *Grippotyphosa* is the most common cause of disease in dogs in the United States (Ward et al., 2004; Stokes et al., 2007), there are nine other serogroups of the species *L.*

kirschneri (Levett, 2001) that could have given a positive PCR in this study. Voles, raccoons, skunks and opossums are implicated as maintenance hosts of grippotyphosa in several countries, but the hosts which may serve as reservoirs in Brazil have not been defined. Some of the dogs on the island with leptospiremia caused by *L. kirschneri* had clinical signs, suggesting that they are probably incidental hosts of the infective serovar.

Certain alterations found on the CBC are “classically” associated with leptospirosis, such as leukocytosis, neutrophilia and anemia (Greene et al., 2006). However, in this study and in other studies a normal hemogram did not exclude the diagnosis of leptospirosis. We also found leukocytosis and anemia in several dogs with negative PCR and serology. The high percentage of anemic dogs from Arquipélago and Veterinary Hospital could be attributed to malnutrition, gastrointestinal parasites, blood parasites and many diseases other than leptospirosis.

Increased serum creatinine is found in dogs with varying severity of renal failure in leptospirosis (Birnbaum et al., 1998; Goldstein et al., 2006; Greene et al., 2006; Ortega-Pacheco et al., 2008). Elevated creatinine levels and clinical signs of icterus were associated with leptospirosis in dogs from the Veterinary Hospital as anticipated in this study, however many dogs with leptospiremia, leptospiruria or that were serologically positive in the other groups, had a normal creatinine. Glucosuria was positively associated with leptospiruria. However, this finding should not be used as a predictor of leptospiruria, since several dogs with positive PCR in urine had normal urinalysis in the present study and some dogs with glucosuria had a negative PCR in urine.

In conclusion, the results presented in this study support previous studies documenting infection with *canicola*, *icterohaemorrhagiae* and *copenhageni* serovars in dogs in Brazil. Our work suggests that dogs may play a role in the leptospirosis cycle as a reservoir host when

excreting leptospire in their urine, and must be constantly monitored by PCR so that appropriate treatment can be given and to minimize their impact on transmission, especially to human populations. It is important to emphasize that apparently healthy dogs may have leptospiremia or be a silent carrier as observed in several dogs from the Arquipélago and CCZ. If creatinine is elevated, particularly in an icteric dog, the likelihood of leptospirosis must be considered, however normal findings for these parameters does not rule out this diagnosis. *L. kirschneri* infection was documented in the Arquipélago dogs by PCR. Whether or not human infection with *L. kirschneri* is occurring and the possibility that dogs serve as a reservoir for such infections, need to be investigated. Further, the use of an effective vaccine that includes serovars of *L. kirschneri* infecting dogs on the island is indicated.

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Conflict of Interest Statement

No conflict of interest to declare.

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Table 1. Characteristics of the study population

Characteristics	Population		
Source	Arquipélago	CCZ	Veterinary Hospital
Questionnaire	Yes	No	Yes
Source	Pet owned	Shelter dogs	Pet owned
Age	1-12 years	Adult dogs, >1 year	5months-11 years
Gender	71 female	10 female	11 female
	84 males	55 male	22 male
Total	155	65	33

Table 2. Categorical variables used in the statistic analysis.

Variable	Observation	Number of categories
Animal characteristics		
Gender	253	Female, male
Group source	253	CCZ, Arquipélago, Veterinary Hospital
Breed	188	Mixed, others
Blood analysis		
PCV	253	Anemic (PCV \leq 36%), normal (PCV $>$ 36%)
ALT	253	Normal ($<$ 102.0 U/L), high ($>$ 103.0 U/L)
Creatinine		Normal (\leq 1.5 mg/dL), high ($>$ 1.5 mg/dL)
WBC		Normal (6,000 – 1,7000), leukocytosis ($>$ 17000)
PCR	253	Negative, Positive
Serology	253	Negative, Positive
Urine analysis		
PCR	253	Negative, Positive
Glucose	234	No, yes
Bilirubin	233	No, +, ++, +++
Blood	234	No, +, ++, +++
Protein	234	No, +, ++, +++
Epithelial cells	228	No, yes
Leukocytes	228	No, +, ++, +++
Hematuria	228	No, +, ++, +++
Casts	228	No, yes
Specific gravity	253	Isosthenuria ($<$ 1.015), normal(1.015 – 1.045) , Hypersthenuria ($>$ 1.045)
pH	233	Suboptimal to leptospire survival(\leq 6.5), optimal ($>$ 6.5)
Clinical signs		
Icterus	188	No, yes
Diarrhea	188	No, yes
Weight loss	188	No, yes
Body condition	188	Normal, skinny, cachexia
Polyuria/ polydipsia	188	No, yes
Vomiting	187	No, yes
Hypotermia/Fever	22	Hypotermia ($<$ 37.5°C), normal (37.5-39.3°C), Fever ($>$ 39.3°C)
Potential risk factors		
Environment	188	Indoor, outdoor
Street access	188	No, yes
Vaccinated	188	No, within one year, more than one year
Presence of rats	188	No, yes

Contact with other animals 188

No, contact with dogs (only), contact with dogs and horses*

*Because only a few dogs had contact with cats, and these dogs had contact with dogs and horses too, the data about cats was not included in the analysis

Table 3. PCR and serology results of the three studied populations.

Group	<u>PCR in blood</u>		<u>PCR in urine</u>		<u>Serology</u>		Total of dogs in each group
	Positive	Negative	Positive	Negative	Positive	Negative	
CCZ	5 ^a (7.7%)	60 (92.3%)	13 ^c (20.0%)	52 (80.0%)	35 ^e (53.8%)	30 (46.2%)	65 (100%)
Arquipélago	23 ^{a,b} (14.8%)	132 (85.2%)	13 ^d (8.4%)	142 (91.6%)	63 ^f (40.6%)	92 (59.4%)	155 (100%)
Veterinary Hospital	9 ^b (27.3%)	24 (72.7%)	10 ^c (30.3%)	23 (69.7%)	24 ^e (72.7%)	9 (27.3%)	33 (100%)
Total	37 (14.6%)	216 (85.4%)	36 (14.2%)	217 (85.8%)	122 (48.2%)	131 (51.8%)	253 (100%)

Same letters means no difference between groups.

4.2. ARTIGO 2

Diagnosis and doxycycline treatment follow-up of leptospiuria in four naturally infected dogs

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SUMMARY

The efficacy of doxycycline for clear leptospires from urine in four asymptomatic naturally infected dogs was investigated. Three dogs were infected with the serogroup Canicola and one dog with the serogroup Icterohaemorrhagiae. Doxycycline (5 mg/kg twice a day) was administered for two weeks. The dogs were followed until 30 days after the beginning of treatment. None of them had side effects. Count blood cells was unremarkable; serum biochemistry and urinalysis were according to the reference species range along the treatment period and serology for leptospirosis had negative or low titers in three of the four dogs. Doxycycline was effective to clear

leptospirosis in all dogs of this study; the absence of leptospires in the urine samples was confirmed by three serial negative PCR results in each dog.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic bacteria of the genus *Leptospira*. Infection by host-adapted species may result in subclinical infection and hosts may intermittently shed leptospires through their urine, spreading the agent and acting as reservoirs. Dogs are reportedly reservoirs of serovars canicola and bataviae, and most likely of bratislava (Lappin 2003), and incidental hosts for other serovars (Burr and others 2009). In addition, dogs have shown leptospirosis after experimental infection with serovar icterohaemorrhagiae (Klaasen and others 2003, Schreiber and others 2005) and serovar copenhageni have been detected in kidneys (Yasuda and Santa Rosa 1981, Cordeiro and Sulzer 1983) and urine (Rodrigues 2008) of naturally infected dogs in Brazil

When leptospirosis is associated with clinical signs, the target organs are the kidney and the liver, depending on the serovar (Burr and others 2009). Carrier dogs may develop renal failure as a result of tubular damage associated with colonization and replication of the leptospires in epithelial cells of the renal tubules (Langston and Heuter 2003). Asymptomatic leptospirotic dogs are mostly diagnosed during epidemiological surveys (Harkin and others 2003), or when an owner (Feigin and others 1973) or another dog in the same household present overt disease compatible with leptospirosis.

Detection of leptospires in urine may be accessed by darkfield microscopy, organism culture or molecular techniques such as polymerase chain reaction (PCR). Darkfield microscopy has reportedly shown a low sensitivity and specificity, whereas bacterial culture has low sensitivity and is time consuming. PCR may be a more sensitive and useful technique to detect the presence of leptospires in blood, urine and

tissues (Ahmad and others 2005). Multilocus variable-number tandem-repeat (VNTR) analysis is a molecular technique used for DNA fingerprint, and can be useful for identifying serovars (Salaun and others 2006). Leptospirosis detection and monitoring in dogs is not routinely used in veterinary clinics and hospitals, and therefore zoonotic potential due to *Leptospira* shedding in urine is still to be fully established.

Regardless of clinical signs, dogs with leptospiremia/leptospirosis should undergo antimicrobials treatment to clear the bacteria from the organs, especially the kidneys, eliminating the state of renal carrier and the zoonotic potential (Murray and Hospenthal 2004). Antimicrobial drugs may not eliminate leptospiral infections in all carrier species, particularly dog infected with serovar canicola (Van de Maele and others 2008). A major limitation on leptospirosis/leptospirosis studies is that those are mostly performed in a short term period (6 to 21 days), and using hamsters as animal models (Truccolo and others 2002, Moon and others 2006, Griffith and others 2007).

Human cases of leptospirosis caused by serovar icterohaemorrhagiae have been reported in which the companion dogs, naturally infected, were implicated as the probable source of infection (Feigin and others 1973). Therefore, asymptomatic dogs shedding leptospire in their urine may act as silent disseminators of infection to humans and other animals sharing the same environment. Doxycycline has been successfully indicated for leptospirosis treatment (Langston and Heuter 2003), mostly due to its lipid-solubility, well absorbance in body tissues, high levels in kidney and liver, and predominantly excretion in faeces. A study in dogs indicated a half-life of 10.36 h, low clearance, good distribution volume and plasmatic detection for 48 h (Wilson and others 1988).

Although leptospirosis carrier state may be cleared in dogs with a dose of 5 mg/kg twice a day for two weeks (Langston and Heuter 2003, Greene and others 2006),

no study has been performed to date on long term monitoring of naturally occurring cases following treatment. Accordingly, this study was undertaken to verify the efficacy of standard dose of doxycycline in effectively clear leptospirosis in four naturally infected asymptomatic dogs.

MATERIALS AND METHODS

The present study has been approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul, Brazil.

Dogs

During an investigative survey of leptospirosis in dogs in Porto Alegre, Brazil, in 2008, four clinically healthy mixed-breed adult male dogs detected with leptospirosis were submitted to doxycycline treatment. Three of these dogs (dogs 1, 2, and 3) lived together in a shelter pen, and the fourth (dog 4) was an owned pet. The definitive diagnosis was based on a positive PCR results in urine.

Dogs were orally given doxycycline at dose of 5 mg/kg twice a day for two weeks. Shelter dogs were kept in same cage and treated by a director veterinarian; owned dog was kept at home and medicine administered by the owner.

Samples were collected at days -10, 0 (only for PCR and darkfield microscopy), 8, 18 and 30. CBC and urinalysis were performed at days -10, 8, 18 and 30 of antibiotic treatment. Laboratorial exams (CBC, urinalysis, serum biochemistry, darkfield microscopy, PCR and serology) were performed regularly until two weeks after finish doxycycline treatment. Variable number tandem repeat (VNTR) was performed in the samples of day -10 of treatment. Clinical examination was daily performed in shelter dogs and the owned dog was weekly evaluated. The owner received orientation about

the zoonotic risk of the disease, proper care and immediate contact in case of any sign of disease.

Haematology, urinalysis and serum biochemistry

Complete blood count (CBC) was performed using a semiautomatic cell counter (CC 530, CELM, Sao Paulo, Brazil), with exception of packed cell volume (PCV) which was obtained by microhaematocrit method. Specific leukocyte differentials were performed on blood smear by light microscopy. Serum creatinine, urea, alanine aminotransferase, alkaline phosphatase, gamma glutamyl transferase, calcium, phosphorus, albumin and total protein were performed by spectrophotometry; potassium was measured by dry chemical analysis. Urinalysis was performed following standard protocols.

Darkfield microscopy, PCR and serology

Direct examination of spirochetes under darkfield microscopy was performed in fresh serum and urine. A drop of sample was observed in 200 and 400× magnification, to detect the presence of one or more spirochetes/field.

For blood PCR analysis, DNA was extracted using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocol. DNA extraction from urine samples was performed in duplicate, using a previous reported protocol (Lucchesi and others 2004), with few modifications: PBS pH 7.4, non-refrigerated centrifuge, and final pellet resuspended in PBS. The primer sets G1/G2 and B64-I/B64-II (Gravekamp and others 1993) were used in singleplex assays. G1/G2 amplifies DNA from strains of *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchi*, *L. santarosai*, and *L. meyeri*, whereas B64-I/B64-II amplifies DNA from *L. kirschneri*. G1/G2 generates a 285-bp and B64I/B64I a 563-bp product. Electrophoresis of 15 µL

of the reaction solution was performed in agarose gel with 0.1 g/mL ethidium bromide, and visualization and photography of the bands of the expected size products were performed under UV light. To identify the serovar of *Leptospira*, a molecular typing system based on multilocus variable-number tandem-repeat (VNTR) analysis (Salaun and others 2006) was performed in DNA extracted from urine.

Serology was performed by microscopic agglutination test (MAT), to detect the presence of antibodies against *Leptospira* antigens, considering positives titers >1:100. The tested serovars included australis, autumnalis, bratislava, canicola, copenhageni, grippotyphosa, hardjo, hebdomadis, icterohaemorrhagiae, pomona, pyrogenes, tarassovi and wolffi.

RESULTS

Results of antibodies and leptospirae detection during the time of evaluation are shown in Table 1.

Spirochetes were observed in the urine of dogs 1, 2 and 4 by darkfield microscopy at days -10 and 0 of treatment. Dog 3 showed negative results by darkfield microscopy in all collections, but leptospiuria was confirmed by PCR. Spirochetes were not observed in serum of any of the animals during the analyzed period.

A 285 bp fragment was amplified from urine samples (leptospiuria) from all dogs using the primers G1/G2. Dogs 1 and 2 had a concurrent positive PCR in blood DNA (leptospiemia), using the same pair of primers. No amplification was obtained with the primers B64-I/B64-II, indicating that the species *L. kirschneri* was not present.

PCR products were obtained from amplification of VNTR 4, VNTR 7 and VNTR 10 loci in the urine of all dogs. For dogs 1, 2 and 3, the deduced serogroup was Canicola (copy number of tandem repeats were compatible with serovars canicola or

portlandvere), species *L. interrogans*. For dog 4, analysis of the urine DNA generated products size compatible with species *L. interrogans* serogroup Icterohaemorrhagiae (serovar copenhageni or icterohaemorrhagiae).

The serology results showed that dog 1 had low positive titers (1:100) for several serovars throughout the evaluation period. In contrast to the VNTR analysis, canicola positive titers only appeared at the day 8 following antibiotic treatment in this animal. Dog 2 showed a unique low seroreactivity for canicola at day -10, which is in agreement with the VNTR analysis. Low positive titers for multiple serovars were then detected during the antibiotic treatment follow-up. Dog 3, on the other hand, was negative at the first evaluation (day -10) but also showed multiple serovars positivity (1:100), including canicola, in the subsequent blood collections. Dog 4 showed a high titer for serovar copenhageni (1:1,600) at day -10, also in agreement with VNTR analysis, and low titers (1:100) for multiple serovars in the following blood collections.

CBC showed results within the reference range for dogs 1, 2 and 3 in all blood collections. Dog 4 had mild leukocytosis in all blood analysis (21,000- 26,500 cells/ μ L), followed by eosinophilia, monocytosis and lymphocytosis. Serum biochemistry and urinalysis had values in the range of reference in all dogs during all the time of evaluation.

No clinical signs of disease were observed during the evaluation period.

DISCUSSION

Doxycycline was effective in clearing the leptospire from urine in all 4 naturally-infected dogs in this study. Although studies have shown that this antibiotic is effective to clear leptospire from urine of human beings (McClain and others 1984) and from kidneys of experimentally infected hamster (Trucollo and others 2002), it was

found only a study that reported a negative PCR in urine after the treatment with doxycycline, in one naturally infected dog (Harkin and others 2003a). Dihydrostreptomycin is another possibility of antibiotic to clear renal carriage, however it is an aminoglycoside and should not be use in dogs with renal damage; in addition, it needs to be administered intramuscularly twice a day for two weeks (McDonough 2001) (not having advantages compared to doxycycline). It is noteworthy that doxycycline did not show any short-term side effects in the treated animals herein.

It was important to obtain (three) serial negative PCR results to suggest clearance, since leptospiuria could be intermittent, principally considering that the dogs might carry serovars for which they were adapted and that a few number of leptospire might be excreted in the urine. VNTR analysis is not able to distinguish between serovar icterohaemorrhagiae and copenhageni, but the high serological titer to serovar copenhageni (1:1,600) in dog 4 suggested that the copenhageni was the serovar infecting this dog. Although dogs are not considered the maintenance hosts of serogroup Icterohaemorrhagiae, this dog did not develop the expected clinical signs related to liver disease as icterus and vomiting, and thereby was a silent carrier. The only alteration noticed was a mild leukocytosis during all the observation time, but it could be associated with other concurrent undiagnosed disease. Although serology by MAT is the most common method used to diagnosis leptospirosis in dogs (Sessions and Greene 2004), in the present study showed several limitations, also described by others authors: negative serology despite of leptospiuria (Van den Broek and others 1991, Yasuda and Santa Rosa 1981) in dog 3; multiple positive titers indicating cross-reaction (Levett 2001, Sessions and Greene 2004); and failure to detect a rising titer in paired samples, probably due to the antibiotic therapy (Burr and others 2009). In a study with human patients comparing serology and culture for serovar identification, MAT had

little value for identification of the infecting serovar and also for the presumptive serogroup (Levett 2003). The fact of dog 3 have had negative serology suggests an adaptation of the harbour serovar.

Dogs 1 and 2 had concurrent leptospiremia and leptospiruria, indicating that they were at the end of the acute stage of the disease, which lasts approximately 10 days (van de Maele 2008). In the beginning of the treatment (day 0), they did not have leptospiremia although contaminating the environment through leptospire excretion in urine. Dog 3 was a possible source of contamination for dogs 1 and 2 in the shelter where they were living together before this study. Besides, his habit of urinating in the drinking water was some evidence that he had been infected before the other two dogs, as a negative PCR in blood but a positive PCR in urine with negative serology. According to Langston and Heuter (2003), dogs harbouring serovar canicola in their kidneys could have leptospiruria and titer <1:100.

Although high antibody titers or increased paired titer on MAT associated with compatible clinical signs and clinicopathological abnormalities support the diagnosis of canine leptospirosis, a negative or low titer did not discard the infection (Burr and others 2008). Considering all the information above, dogs 1, 2 and 3 could be misdiagnosed as free of leptospirosis, if techniques to detect the presence of leptospire were not performed. In that case, a misinterpretation of a dog without leptospirosis and thereby not representing a risk for humans and other dogs could occur. PCR was essential for the correct diagnosis in all dogs; darkfield microscopy omitted leptospiruria in one dog and leptospiremia in the 2 dogs diagnosed by PCR. On the other hand, although relevant clinicopathological alterations were not detect, urinalysis and serum biochemistry are useful to certify that the dog does not develop hepatic or

renal alterations, because a specific treatment, as fluid therapy and other supportive treatment may be necessary.

The results confirmed that doxycycline was efficient to clear leptospiruria in dogs. The outcome of a dog with leptospiremia or leptospiruria should be performed using molecular techniques that identify the leptospire, independent of clinical signs or clinicopathological findings.

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Table 1. Serology, dark field microscopy and molecular analysis following doxycyclin treatment in leptospiruric dogs.

Analysis	Dog	Day					Molecular typing
		-10	0	8	18	30	
Serology (titer and serovar)	1	1:100 (cop, ict, pom)*	NP	1:100 (can, cop, ict, pom, wol)*	1:100 (can, cop, pom, wol)*	1:100 (can, cop, ict, pom, wol)*	Serogroup Canicola
	2	1:100 (can)	NP	1:100 (can, cop, ict, wol)*	1:100 (can, cop, ict, wol)*	1:100 (can, cop, ict, wol)*	Serogroup Canicola
	3	-	NP	1:100 (can, cop, ict, pom)*	1:100 (can, cop, ict, pom, wol)*	1:100 (can, cop, ict, pom, wol)*	Serogroup Canicola
	4	1:1,600 (cop),	NP	1:100 (aut, cop, ict)*	1:100 (cop, ict)*	1:100 (cop, ict)*	Serogroup Icterohaemorrhagiae
Darkfield microscopy	1	+ (urine)	+ (urine)	-	-	-	
	2	+ (urine)	+ (urine)	-	-	-	
	3	-	-	-	-	-	
	4	+ (urine)	+ (urine)	-	-	-	
PCR	1	+ (blood and urine)	+ (urine)	-	-	-	
	2	+ (blood and urine)	+ (urine)	-	-	-	
	3	+ (urine)	+ (urine)	-	-	-	
	4	+ (urine)	+ (urine)	-	-	-	

+ (positive), -(negative), NP (not performed); aut (autumnalis), can (canicola), cop (copenhageni), ict (icterohaemorrhagiae), pom (pomona), pyr (pyrogenes), wol (wolffi).

*Cross-reaction.

1 **4.3. ARTIGO 3**

2

3 **Serum and urinary C-reactive protein concentrations in dogs with leptospirosis***

4

5 Concentrações de proteína C-reativa sérica e urinária em cães com leptospirose

6

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10

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11 **ABSTRACT**

12 Leptospirosis is a bacterial disease caused by pathogenic strains of *Leptospira*, which may
13 affect human beings and a wide range of both domestic and wild animals. Clinical signs and
14 clinicopathologic alterations depend on the virulence of the organism and host susceptibility. The
15 disease in dogs is still a challenge for clinicians, since definitive diagnosis may be reached only
16 few days after overt clinical signs. Besides, dogs with leptospirosis represent a zoonotic risk,
17 making development of rapid screening tests crucial for early diagnosis of disease. C-reactive
18 protein is a positive acute phase protein, and in the dog a strong and fast response is expected
19 after any tissue injury. Acute phase proteins are considered early markers of inflammatory or
20 infectious process, and can be detected before stimulation of the specific immune response.
21 Accordingly, the aim of this study was to evaluate serum and urinary C-reactive protein as
22 potential early indicators of leptospirosis in dogs, and its association with clinical serum
23 biochemistry, complete blood count (CBC) and clinical outcome. A total of 62 suspicious dogs
24 with risk factors and/or clinical signs of leptospirosis were prospectively obtained and included in
25 this study. Definitive diagnosis was based on serology, using the microscopic agglutination test
26 (MAT) against 13 serovars, and on a specific polymerase chain reaction (PCR) in blood or urine,
27 using the primers sets G1/G2 and B64-I/B64-II, which amplify DNA of pathogenic leptospires.
28 Clinical serum biochemistry included creatinine, urea, alanine aminotransferase, alkaline
29 phosphatase, creatine kinase and albumin. C-reactive protein was performed in serum and urine
30 using a semi-quantitative latex-agglutination test. A total of 49/62 (79%) dogs presented a
31 positive diagnosis of leptospirosis. From these, 12 (24.5%) had positive blood PCR, 17 (34.7%)
32 positive urine PCR and 43 (87.7%) had positive serology. Concurrent positive serology and
33 positive PCR (blood or urine) occurred in 19 (38.8%) dogs, whereas 24 (49%) dogs had positive
34 serology only, and 6 (12.2%) dogs had positive PCR only. Dogs with negative results at serology

35 and PCR were kept for analysis and participated as negative control group. Out of the 62 dogs, 25
36 (40.3%) had high liver enzymes, 18 (29%) had azotemia, 23 (37.1%) had leukocytosis, 37
37 (59.7%) had high creatine kinase levels and 37 (59.7%) had hypoalbuminemia. Twelve death
38 cases (19.3%) occurred within 10 days after the sample collection. Positive serology was
39 significantly associated with urinary C-reactive protein (P= 0.038). However, only a weak
40 association was found between serum C-reactive protein and blood PCR (area under curve=
41 0.68). There was no association between urinary C-reactive protein and urine PCR, urinary C-
42 reactive protein and blood PCR, serum C-reactive protein and positive serology, or serum C-
43 reactive protein and urine PCR. Increased liver enzymes (P=0.04) and hypoalbuminemia
44 (P=0.002) were associated with high levels of serum C-reactive protein. There was no association
45 between serum or urinary C-reactive protein and obit. In conclusion, although C-reactive protein
46 may be used as part of a screening profile, it should not be considered as indicator alone of
47 leptospirosis screening in dogs.

48 **KEY WORDS:** dog, leptospirosis, diagnosis, serology, PCR, screening, C-reactive protein.

49

50 **INTRODUCTION**

51 Leptospirosis is a zoonotic disease caused by spirochetes of the genus *Leptospira*. The
52 disease is biphasic, with a phase of leptospiremia in the first week, followed by a second phase of
53 antibody production and initiation of *Leptospira* shedding into urine (leptospiuria) [2]. The
54 infection in dogs does not always result in overt clinical signs [11], being dependent on the
55 organism virulence and host susceptibility [8]. Definitive diagnosis is based on detection of
56 serum antibodies (serology) or detection of leptospire in clinical material (molecular techniques
57 and/or organism culture/isolation) [11].

58 C-reactive protein (C-RP) is a positive acute phase protein (APP) produced in
59 hepatocytes (although human renal cortical tubular epithelial cells are also able to produce C-RP)
60 and released into the bloodstream as consequence of inflammatory or infectious processes [7].
61 APPs have a rapid serum production and clearance, mostly reflecting the dog *status* at the time of
62 sampling. Therefore, APPs may be useful to provide evidence of acute inflammatory or
63 infectious disease, even with no evident clinical signs; however, it should not be used as a
64 primary diagnostic test due to its poor specificity [4].

65 The aim of this study was to verify the usefulness of C-reactive protein (C-RP) levels in
66 serum and urine as an early indicator of leptospirosis in dogs. In addition, we investigated the
67 possible association between C-RP and clinical serum biochemistry, complete blood count (CBC)
68 and clinical outcome in these dogs.

69

70 **MATERIALS AND METHODS**

71 Suspicious dogs from either the Veterinary Teaching Hospital (Federal University of Rio
72 Grande do Sul) or the Control Center of Zoonoses at Porto Alegre, Brazil were screened,
73 evaluated (between August of 2007 and February of 2008) and included in the present study.
74 Suspicious dogs should have risk factors (history of rats and/or lacking of vaccination associated
75 with outdoor access) and/or clinical signs (icterus, anorexia/weight loss, vomiting, diarrhea,
76 polyuria/polydispsia and fever) of leptospirosis, with no history of antibiotic treatment. Definitive
77 diagnosis was confirmed by either serology titer $\geq 1:100$ for any of the tested serovars, or positive
78 PCR in blood or urine. Dogs with negative results were kept for analysis and participated as
79 negative control group. The present study has been submitted and approved by the University
80 Ethics Committee, following ethical principles for animal experimentation of the Brazilian
81 College of Animal Experimentation (COBEA).

82 Serology was performed using the Microscopic Agglutination Test (MAT) under
83 standard protocol, and tested against the following serovars: australis, autumnalis, bratislava,
84 canicola, copenhageni, grippityphosa, hardjo, hebdomadis, icterohaemorrhagiae, pomona,
85 pyrogenes, tarassovi and wolffi. Titers $\geq 1:100$ were considered positives.

86 Blood DNA was extracted using a commercial kit following the manufacturer's
87 protocol¹. Urine DNA extraction was performed using a modified protocol [9]. Briefly, PBS (pH
88 7.4) was added to urine in a 1:2.5 v/v proportion to neutralize the sample immediately after
89 collection. Urine DNA extraction was performed in duplicate. A total of 1.5 mL of the sample
90 was incubated at 37°C for 10 minutes, and then centrifuged at 800×g at room temperature. The
91 supernatant was centrifuged at 1560×g for 20 minutes. The resultant supernatant was discharged;
92 pellet was resuspended and washed with 1 mL of PBS, and then centrifuged at 1560×g for
93 additional 20 minutes. The supernatant was discharged and the pellet was resuspended in 100 μ L
94 of PBS and boiled for 10 minutes. DNA was stored at -20°C until molecular analysis.

95 The primer sets G1/G2 and B64-I/B64II [5] were used to amplify DNA from pathogenic
96 leptospire in blood (leptospiemia) and urine (leptospiuria). PCR reactions were carried out in
97 1× buffer, 1.5 mM MgCl₂, 0.2 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 5 pmol
98 of each primer, 1.25 U of *Taq* DNA polymerase², 5 μ L of the DNA template and ultrapure water
99 up to 25 μ L. A positive control and two negatives controls were included in each run. The
100 thermocycler conditions consisted of an initial step at 95°C for 2 minutes, followed by 40 cycles
101 of denaturation at 94°C for 1 minute, annealing at 55°C for 45 seconds and extension at 72°C for
102 45 seconds, and a final extension at 72°C for 5 minutes. Electrophoresis (1 h at 100 V) was
103 performed with 15 μ L of the reaction solution in a 1.5% agarose gel with ethidium bromide. PCR
104 products were visualized under UV light and photographed.

105 C-RP analysis was performed in serum and fresh urine supernatant (with no additive)
106 using a semi-quantitative latex-agglutination test³, previous validated for use in canine serum
107 [12]. Results are reported as an approximate mg/L concentration of C-RP. Sensitivity of detection
108 was 6 mg/L.

109 Clinical serum biochemistry was performed by spectrophotometry in a semi-automatic
110 analyzer⁴ using commercial kits⁵, and included creatinine, urea, alanine aminotransferase,
111 alkaline phosphatase, creatine kinase and albumin. Complete Blood Count (CBC) was performed
112 in EDTA blood using a semiautomatic cell counter⁶.

113 Chi-square test or Fisher's exact test (when appropriate) were used to investigate the
114 association between both serum and urine C-RP and blood and urine PCRs, serology, liver
115 enzymes, azotemia, leukocytes count, creatine kinase and albumin concentrations, and animal's
116 death. Receiver operator curve (ROC) was also plotted for serum and urine C-RP using blood and
117 urine PCRs as a reference test. A p-value <0.05 was considered significant. Stata 11 (StataCorp,
118 College Station, TX) was used for the analysis.

119

120 **RESULTS**

121 Sixty-two suspicious dogs were preliminary screened and evaluated. A total of 49/62
122 (79%) dogs were positive for leptospirosis based on serology and/or PCR. From these, 12
123 (24.5%) had positive blood PCR, 17 (34.7%) positive urine PCR and 43 (87.7%) had positive
124 serology. Concurrent positive serology and positive PCR (blood or urine) occurred in 19 (38.8%)
125 dogs, whereas 24 (49%) dogs had positive serology only, and 6 (12.2%) dogs had positive PCR
126 only. Out of the 62 dogs, 25 (40.3%) had high liver enzymes, 18 (29%) had azotemia, 23 (37.1%)
127 had leukocytosis, 37 (59.7%) had high creatine kinase levels and 37 (59.7%) had

128 hypoalbuminemia. Twelve death cases (19.3%) occurred within 10 days after the sample
129 collection.

130 Serum and urinary C-RP concentrations in dogs with leptospirosis and in negative dogs
131 are shown in Table 1. Positive serology was associated with urinary C-RP ($P= 0.038$), but not
132 with serum C-RP. There was a weak association between serum C-RP and blood PCR (Fig. 1)
133 whereas no association was found between serum C-RP and urine PCR; between urinary C-RP
134 and blood PCR; or between urinary C-RP and urine PCR. Increased liver enzymes ($P=0.04$) and
135 hypoalbuminemia ($P=0.002$) were significantly associated with serum C-RP (data not shown).

136

137 **DISCUSSION**

138 Higher concentrations of serum C-RP in dogs were reported in infectious diseases when
139 compared to other causes, despite of its non-specificity [13]. Therefore, increased blood C-RP
140 may be expected in dogs with leptospiremia, and increased urinary C-RP may be expected in
141 leptospiruria. However, our results have shown that serum C-RP was unable to predict
142 leptospiremia, and no relationship between urine PCR and urinary C-RP was found. Although
143 associations between serology and urinary C-RP levels were observed, and the test may be
144 included in a diagnostic screening profile, our data suggest that serum and urinary C-RP should
145 not be used as screening test alone for leptospirosis in naturally infected dogs.

146 In the present study, association between serum C-RP and serology was not observed, and
147 the association of urinary C-RP with serology does not justify its use, since the urinary C-RP test
148 did not detect dogs with leptospiremia or leptospiruria. Interestingly, previous studies have
149 shown that serum C-RP has increased up to 16 to 80-fold [3] in five experimentally and 30-fold
150 in two naturally infected dogs with leptospirosis [13]. The authors hypothesize that *Leptospira*

151 strains may have different pathogenicity among studies, or that previous studies were conducted
152 with small sample size.

153 Typical clinicopathological abnormalities in dogs with leptospirosis include
154 leukocytosis, increased blood liver enzymes and bilirubinemia in cases of hepatic damage, and
155 increased concentrations of blood urea and creatinine if renal insufficiency is present [1,5,10].
156 Decreased serum albumin levels and high creatine kinase levels are also common findings [8].
157 Albumin is a negative APP, and as C-RP, the change in its concentrations may occur in different
158 inflammatory or infectious conditions. Thereby, the association between C-RP and albumin that
159 occurred in the present study was expected, independent of the primary disease. Since association
160 of C-RP with high levels of hepatic enzymes was weak, C-RP may be used as part of a screening
161 profile, but not as indicator itself for either hepatic diseases of leptospiral infection.

162

163 **CONCLUSIONS**

164 Although C-RP may be used as part of a screening profile, it should not be consider as indicator
165 alone of leptospirosis screening in dogs, since no advantage was observed including C-RP as a
166 screening test in dogs suspected of leptospirosis.

167

168 **SOURCES AND MANUFACTURERS**

- 169 1. QIAamp DNA Blood Mini kit, QIAGEN, Valencia, CA, USA
- 170 2. GoTaqFlexi DNA Polymerase, Promega, Madison, WI, USA
- 171 3. Human GMBH, Max-Planck-Ring 21 D 65205 Wiesbaden, Germany.
- 172 4. Metrolab 1600, Buenos Aires, Argentina
- 173 5. Labtest, Lagoa Santa, MG, Brazil
- 174 6. Celm CC 530, Brazil

175

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179

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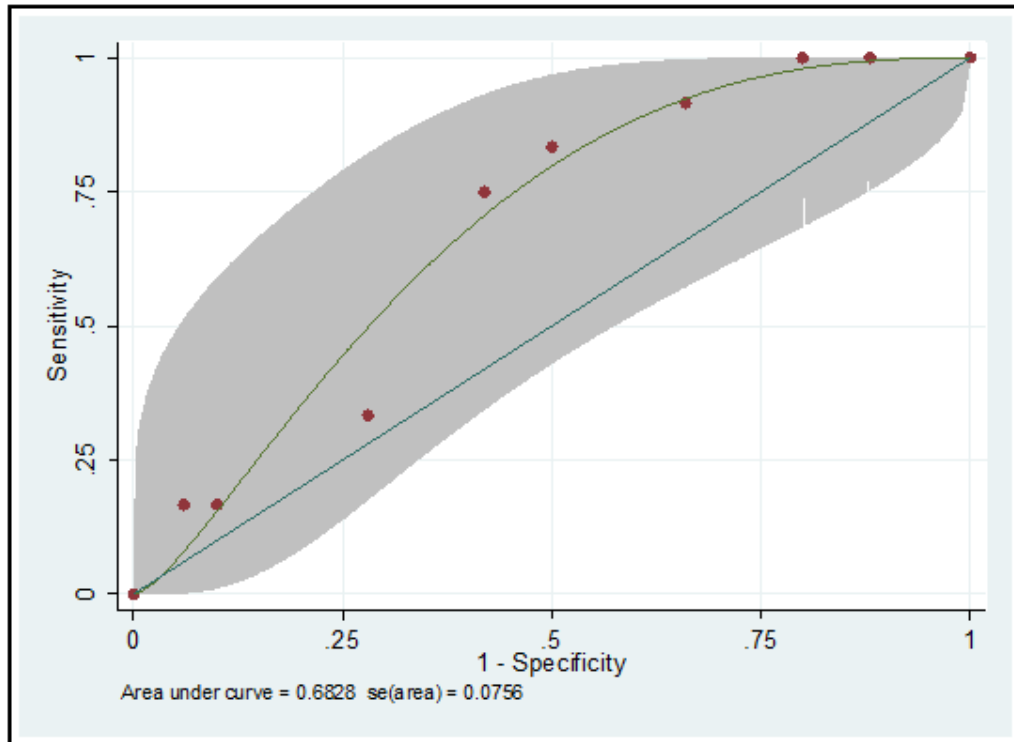
221 Table 1. Serum and urinary C-reactive protein concentrations in 62 dogs with or without leptospirosis,
 222 diagnosed by MAT and PCR in blood and urine.

Diagnosis of leptospirosis	n. of dogs	Serum C-reactive protein			Urinary C-reactive protein		
		Mean (mg/L)	Range	n. of dogs with C-RP \geq 6	Mean (mg/L)	Range	n. of dogs with C-RP \geq 6
Serology							
Positive	43	84	<6-384	39	13	<6-96	11
Negative	19	133	<6-384	17	34	<6-192	7
Blood PCR (leptospiemia)							
Positive	12	143	12-384	12	29	<6-192	4
Negative	50	89	<6-384	44	17	<6-192	14
Urinary PCR (leptospiruria)							
Positive	17	69	<6-384	15	18	<6-192	3
Negative	45	111	<6-384	41	29	<6-192	15
All methods together *							
Positive	49	96	<6-384	44	19	<6-192	13
Negative	13	112	<6-384	12	20	<6-192	5

223 * Considering all dogs diagnosed, including concurrent positive results and dogs with only one positive result
 224 (serology or blood PCR or urine PCR).

225

Figure 1. Fitted receiver operating characteristic (ROC) for the ability of serum C-PR to predict leptospiremia (positive blood PCR) in dogs, with confidence band.



5. CONSIDERAÇÕES FINAIS

As informações obtidas neste trabalho mostram a importância do diagnóstico, tratamento e prevenção da leptospirose canina. Foram observadas as diferentes formas da apresentação da infecção, assim como a eficácia do tratamento na eliminação do estado de portador renal e possíveis alterações laboratoriais que poderiam auxiliar na triagem dos animais contaminados.

As populações de cães aqui estudadas foram selecionadas por apresentar algumas particularidades, com diferenças entre elas que pudessem ser relevantes no comportamento da doença. O grupo Centro de Controle de Zoonoses (CCZ) foi escolhido por representar a população de cães errantes de Porto Alegre, recolhidos em várias áreas. Apesar de não se possuir histórico prévio destes animais, assume-se que não tenham recebido vacina nos últimos meses, e isto somado ao fato do contato com outros animais e fatores de risco, aumentaria sua chance de contaminação. O grupo Arquipélago representa os cães oriundos principalmente da Ilha dos Marinheiros e da Ilha do Pavão (região de Porto Alegre), que são comunidades pobres cuja principal atividade é a coleta de lixo para reciclagem. Um estudo realizado por Henkes (2008) mostrou que as pessoas residentes no bairro Arquipélago apresentam cinco vezes mais chance de terem leptospirose, em relação à população residente em bairros do estrato socioeconômico alto. Além do baixo poder socioeconômico das pessoas do bairro Arquipélago e o acúmulo de entulho no local, com uma grande população de ratos, o fato de ser uma região alagadiça também contribui para as condições ideais para a manutenção da doença. A maioria dos cães das ilhas, apesar de terem donos, vivem em condições precárias, soltos nas ruas, e convivem com outros animais. O terceiro grupo de cães avaliados, referente aos cães atendidos no Hospital Veterinário, apresenta como particularidade o fato de serem suspeitos da doença por apresentar sinais clínicos compatíveis com leptospirose, ou coabitarem com outro animal com diagnóstico positivo. Este grupo corresponde a uma amostra propositalmente “viciada”, na qual se esperava maior número de cães infectados, com a intenção de compará-la aos outros grupos, tanto em relação à presença de leptospiremia e leptospirúria, quanto em relação a outras alterações clinicopatológicas.

A maioria dos estudos encontrados na literatura se referem a cães sintomáticos, atendidos em hospitais veterinários, e isto justifica os sinais clínicos esperados nestes animais e descritos como “clássicos”, como as alterações renais, hepáticas e hematológicas, incluindo

leucocitose e anemia. Estas alterações também foram verificadas no presente trabalho em relação aos animais que apresentavam sinais clínicos, porém foi verificado que tais alterações não correspondiam necessariamente aos cães infectados, mostrando a importância do diagnóstico específico e definitivo da doença, para que tais animais sejam encaminhados ao tratamento, independente de seu estado aparente de saúde. O fato de vários cães aparentemente saudáveis apresentarem positividade a *Leptospira* no sangue ou na urina, mediante a técnica de PCR, mostra o potencial destes animais de se tornarem carreadores crônicos da leptospirose, contaminando desta forma o ambiente, outros animais e seres humanos.

A técnica de PCR, apesar de recomendada para a detecção das leptospirosas nas amostras de sangue, urina, líquido ou tecidos do paciente, ainda não é amplamente utilizada como diagnóstico de rotina. Esta técnica também apresenta algumas limitações, pois pode apresentar resultados falsos-negativos caso a concentração de leptospirosas na amostra esteja abaixo do nível mínimo detectável (sensibilidade), e neste caso, a PCR em tempo real poderia ser mais adequada, porém seu custo é mais elevado. No primeiro artigo, a comparação entre resultados obtidos através de sorologia e PCR convencional mostra a discrepância dos resultados entre os testes, já observados e explicados na literatura, visto que a produção de anticorpos e a presença da bactéria no indivíduo não ocorrem necessariamente ao mesmo tempo. Os resultados obtidos chamam a atenção para o fato de animais assintomáticos e com exames clinicopatológicos normais apresentarem a infecção.

Em relação ao tratamento, é importante o acompanhamento dos animais durante e após este período, pois os resultados de negatividade em relação à presença das leptospirosas no paciente são úteis, tanto individualmente quanto epidemiologicamente. Como já observado na literatura, alguns antibióticos melhoram o estado clínico do paciente sem no entanto eliminar a bactéria dos tecidos. A avaliação de um grande número de animais naturalmente infectados, submetidos a tratamentos específicos, auxiliaria nesta avaliação da eficácia de cada tratamento, uma vez que possam ocorrer variações quanto ao ambiente, animais e cepas infectantes, dificilmente avaliados artificialmente. Da mesma forma, estudos para a utilização de testes de triagem alternativos, como foi proposto com a proteína C-reativa, são relevantes para a detecção precoce da doença e acompanhamento. O desafio consiste na identificação de marcadores que sejam úteis mesmo nos casos de cães assintomáticos e sem alterações no hemograma, urinálise e exames bioquímicos de rotina. Estes testes não substituem os exames de PCR ou isolamento das leptospirosas para o diagnóstico definitivo, porém auxiliam na

conduta dos clínicos. Infelizmente, apesar da proteína C-reativa ser um exame de baixo custo e poder ser mensurada de forma ambulatorial no caso da aglutinação em látex, o teste não se mostrou útil isoladamente para triagem da leptospirose em cães.

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