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Pathogenic *Leptospira* spp. in bats: Molecular investigation in Southern Brazil

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ABSTRACT

The present study aimed to investigate the frequency of pathogenic *Leptospira* spp. in Brazilian bats and to determine possible risk factors associated to it. Ninety two bats of 12 species were evaluated. Whole genomic DNA from kidneys was extracted and real-time PCR specific to pathogenic *Leptospira* spp. was applied. Association between the frequency of specimens positive for *Leptospira* spp. and sex, age, bat species or family, season of collection, geographic localization and feeding habits was evaluated. The results showed that 39.13% of analyzed bats were found positive for *Leptospira* spp. Nine bat species had at least one positive result. There was no association among the evaluated variables and frequency of pathogenic *Leptospira* spp. Although the limitations due to lack of *Leptospira* spp. isolation, leptospiral carriage was demonstrated in bats of different species from southern Brazil, which reinforces the need for surveillance of infectious agents in wild animals.

1. Introduction

Leptospirosis is a zoonotic disease caused by pathogenic strains of *Leptospira* spp., which colonize host kidneys and are eliminated in urine. The transmission occurs by direct contact with contaminated urine or indirectly through contaminated water or soil [1]. Leptospirosis is worldwide distributed, and its incidence varies with climate, animal reservoirs and surveillance [2]. Rainy periods are associated to higher frequencies of disease, especially in large cities, since there is an increased chance of population contact with contaminated water in inadequate sanitation areas [3].

In Brazil, more than 60,000 human leptospirosis cases were confirmed between 2000 and 2016 and the regions with higher frequency of confirmed cases are the southern and southeastern regions [3]. The higher incidences occur in low-income populations of most populous cities, such as São Paulo or in regions with poor sanitation system [4]. Besides public health issues, leptospirosis is responsible for economic losses for the animal production sector. This is caused by costs with vaccination and decrease of production due to abortion, lower milk production and animal death [1].

An important feature of leptospirosis is the wide host range that are susceptible to disease or can serve as bacteria reservoirs [5], which hinders its control and epidemiologic understanding. Several studies have demonstrated that rodents are the main leptospirosis reservoirs [6]. However, there is a growing knowledge about the role of different wild animal species on disease cycle, which may be important due to their abundance and increasing contact with domestic animals [7,8]. Thus, identifying wild reservoirs is a key factor in leptospirosis epidemiology knowledge [9].

Among *Leptospira* spp. wild reservoirs, bats are being evaluated in different parts of the world with different results according to region [10]. Bats have been implicated in epidemiological cycles of several emerging and re-emerging zoonosis, such as rabies [11], severe acute respiratory syndrome (SARS) [12], leptospirosis [8,13] and recently Ebola in Africa [14], which points them as important key players in the epidemiology of infectious diseases. Bats are found on every continent

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except Antarctica [15]. In Brazil, 178 bat species have been recorded [16], of which 40 are settled in Rio Grande do Sul state, the Brazilian southern region [17]. Despite this wide range of bat species and high rates of leptospirosis, there are few Brazilian studies on this subject showing low bat leptospiral infection rates [4,18]; however, they were performed in urban areas of Southeast region. Thus, in the present study, we sought to investigate the frequency of pathogenic *Leptospira* strains in bats from different areas of Rio Grande do Sul, the southern Brazilian state, which has a different ecosystem.

2. Material and methods

2.1. Ethics statement

Permission for this work on bats was granted by Ethical commission on animal experimentation of the "Instituto de Pesquisas Veterinárias Desidério Finamor" (CEUA/IPVDF) (process number 03/2012). The study did not involve any direct manipulations of live bats and relied entirely on collection of tissue samples from dead bats. All experiments were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series—No. 170 revised 2005) and the procedures of the Brazilian College of Animal Experimentation (COBEA).

2.2. Sampling

Bat samples (n = 92) from 31 and 2 municipalities of Rio Grande do Sul and Santa Catarina states, respectively (Fig. 1) were included in the study. The samples were sent to Instituto de Pesquisas Veterinárias Desidério Finamor for rabies diagnosis between December 2010 and December 2012. Species identification was performed based on previous studies and age determination was made through epiphysis fusion and dentition evaluation [19,20].

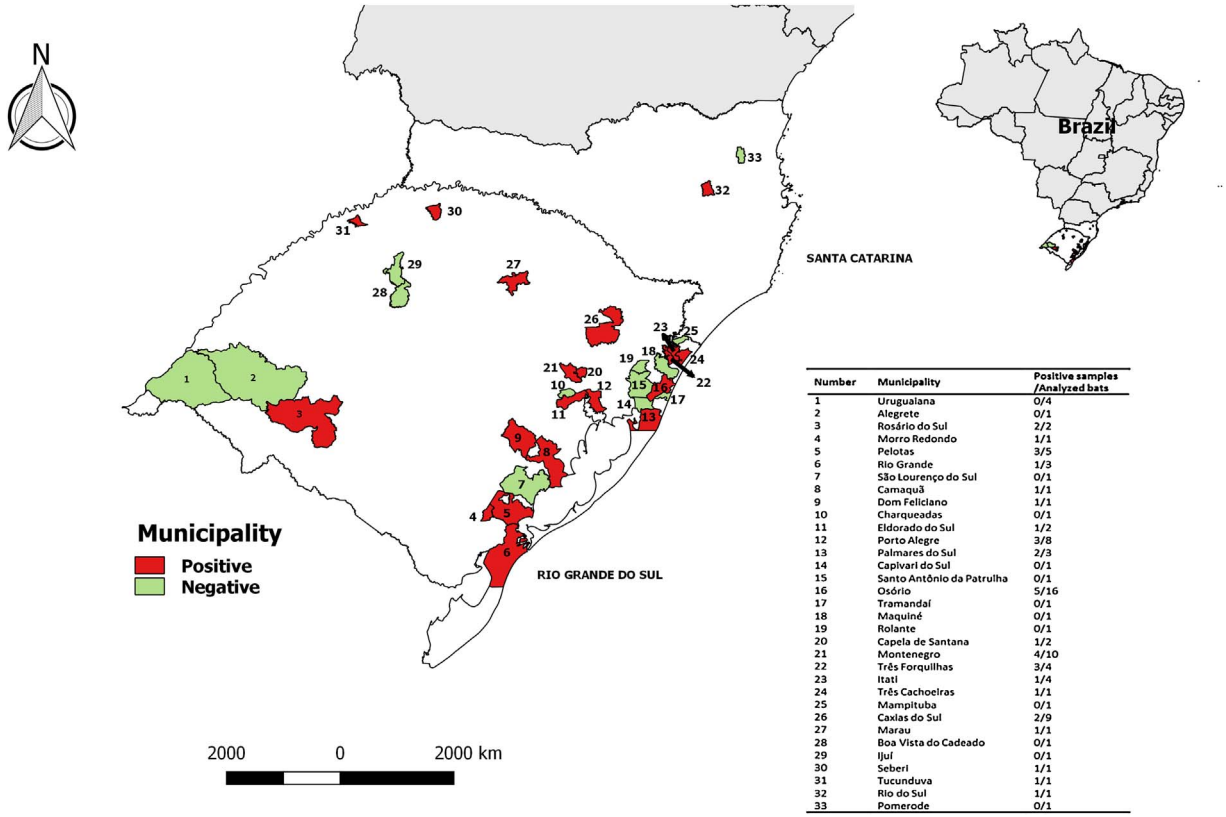
2.3. Molecular detection of pathogenic Leptospira spp

For detection of pathogenic *Leptospira* spp. in bat kidneys, total DNA was extracted as previously described [21] and quantified using a spectrophotometer (L-quant, Loccus Biotechnology, Brazil). A conventional PCR for Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed for DNA quality confirmation [22]. About 25 ng of DNA were used as template for Taqman® real-time PCR with primers and probe targeting *lipL32* as previously published [23]. All reactions were carried out in triplicate and positive results were considered if at least two reactions had detectable CT. Positive and negative controls were included in each run.

To determine the real-time PCR analytical sensitivity, a conventional PCR using the reference isolate Pomona DNA was performed and the resulting amplicon was cloned in a pCR2.1 vector (TOPO TA Cloning® kit, Invitrogen, USA). After *Escherichia coli* DH5-α transformation, plasmid DNA was recovered [24] and quantified with Qubit (Invitrogen, USA). The number of molecules was calculated as follows: $[6.02 \times 10^{23}$ molecules × cloned vector quantity (g)]/Molecular weight of cloned vector (g). Standard curve was made with amounts ranging from 10^{9} to 10^{-1} leptospiral DNA molecules as templates for real-time PCR.

2.4. Statistics

In order to evaluate an association between the positivity frequency for pathogenic *Leptospira* spp. and the dependent variables, chi-square (sex variable) or Fisher Exact test (age, bat species or family, season of collection, geographic localization and feeding habits variables) were



Number	Municipality	Positive samples /Analyzed bats
1	Urugualana	0/4
2	Alegrete	0/1
3	Rosário do Sul	2/2
4	Morro Redondo	1/1
5	Pelotas	3/5
6	Rio Grande	1/3
7	São Lourenço do Sul	0/1
8	Camaquã	1/1
9	Dom Feliciano	1/1
10	Charqueadas	0/1
11	Eldorado do Sul	1/2
12	Porto Alegre	3/8
13	Palmares do Sul	2/3
14	Capivari do Sul	0/1
15	Santo Antônio da Patrulha	0/1
16	Osório	5/16
17	Tramandaí	0/1
18	Maquiné	0/1
19	Rolante	0/1
20	Capela de Santana	1/2
21	Montenegro	4/10
22	Três Forquilhas	3/4
23	Itati	1/4
24	Três Cachoeiras	1/1
25	Mampituba	0/1
26	Caxias do Sul	2/9
27	Marau	1/1
28	Boa Vista do Cadeado	0/1
29	Ijuí	0/1
30	Seberi	1/1
31	Tucunduva	1/1
32	Rio do Sul	1/1
33	Pomerode	0/1

Fig. 1. Geographical distribution of sampled bats. Rio Grande do Sul, the southern Brazilian state is shown. Municipalities in red (n = 20) had at least one bat with positive result for pathogenic *Leptospira* spp. Municipalities in green (n = 13) had at least one evaluated bat and the results were negative. Municipalities in white were not accessed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Description of bat samples collected in Southern Brazil.

Variable		Number of bats (% of total)
Sex	Female	45 (48.9)
	Male	47 (51.1)
Age	Newborn	2 (2.2)
	Juvenile	8 (8.7)
	Adult	76 (82.6)
	Old	3 (3.3)
	Unclassified	3 (3.3)
Species	*Eptesicus diminutus*	3 (3.3)
	Eptesicus furinalis	1 (1.1)
	Eumops auripendulus	5 (5.4)
	Eumops patagonicus	3 (3.3)
	Glossophaga soricina	2 (2.2)
	Histiotus velatus	2 (2.2)
	Lasiurus ega	1 (1.1)
	Molossus molossus	19 (20.7)
	Molossus rufus	8 (8.7)
	Myotis nigricans	1 (1.1)
	Sturnira lilium	1 (1.1)
	Tadarida brasiliensis	46 (50.0)
Season of collection	Spring	39 (42.4)
	Summer	8 (8.7)
	Autumn	4 (4.4)
	Winter	41 (44.6)
Feeding habits	Insectivorous	89 (96.7)
	Nectarivorous	2 (2.2)
	Frugivorous	1 (1.1)
Family	Molossidae	81 (88.0)
	Vespertilionidae	8 (8.7)
	Phyllostomidae	3 (3.3)

performed with Stata software 10.0 (Stata Corporation, College Station, Texas, USA). Statistical association was considered when $p < 0.05$. For geographical representation, Quantum GIS software was used.

3. Results

3.1. Sample characterization

Ninety two bat specimens were analyzed. They were classified into 12 different species, from Phyllostomidae, Molossidae and Vespertilionidae families. Most of the samples were from free tailed bats (*Tadarida brasiliensis*) (Table 1). The majority of animals were adults (82.61%). Sample distribution by sex was homogeneous and the higher frequency of collection occurred during spring and winter (Table 1). The number of analyzed bats by municipality is indicated in Fig. 1.

3.2. Molecular analysis

The real-time PCR was able to detect 10 genome copies of *Leptospira* spp. with 103.42% efficiency ($R^2 = 0.99$) (Fig. 2.), which was the same detection capacity than the previous study [23]. All DNA amplified the *GAPDH* gene, showing that the samples were in appropriate conditions for molecular analysis. Of these, 36 (39.13%) had positive results for pathogenic *Leptospira* (Table 2) and 9 species had at least one positive bat (Table 2).

Among the species with higher number of analyzed specimens, *Tadarida brasiliensis* had almost half of the animals positive for leptospiral genome carriage (21/46; 45.65%; Table 2) and *Molossus molossus* had a lower positivity rate (4/19; 21.05%; Table 2). A low number of other bat species has also been surveyed and their infection rates varied from 0% (*Eptesicus furinalis*, *Eumops patagonicus* and *Sturnira lilium*) to 100% (*Histiotus velatus* (2/2), *Lasiurus ega* (1/1) and *Myotis nigricans* (1/1)) (Table 2).

In 33 municipalities where the samples were collected, 20 had at least, one positive bat (Fig. 1.). Factors such bat species, *Leptospira* spp. serovar, age and breeding season may influence bat leptospiral infection rates. In the present study, there was no association among age ($p = 0.502$), sex ($p = 0.867$), species ($p = 0.139$), season of collection ($p = 0.838$), location ($p = 0.477$), feeding habits ($p = 1.000$) or family ($p = 0.09$) and the frequency of positive results for pathogenic *Leptospira*.

4. Discussion

In the present study, bats from Southern Brazil were shown to be *Leptospira* spp. carriers. The data showed higher frequency than previous Brazilian studies – 39.1% vs 7.8% and 1.75% in Botucatu and São Paulo, respectively [25,26]. This may be related to the environment in which the bats live, as in the present study bats from South Brazilian areas, most of them distant from urban centers, were evaluated.

A variable number of bats from the surveyed species were analyzed; however, it was possible to observe an equal distribution of positive and

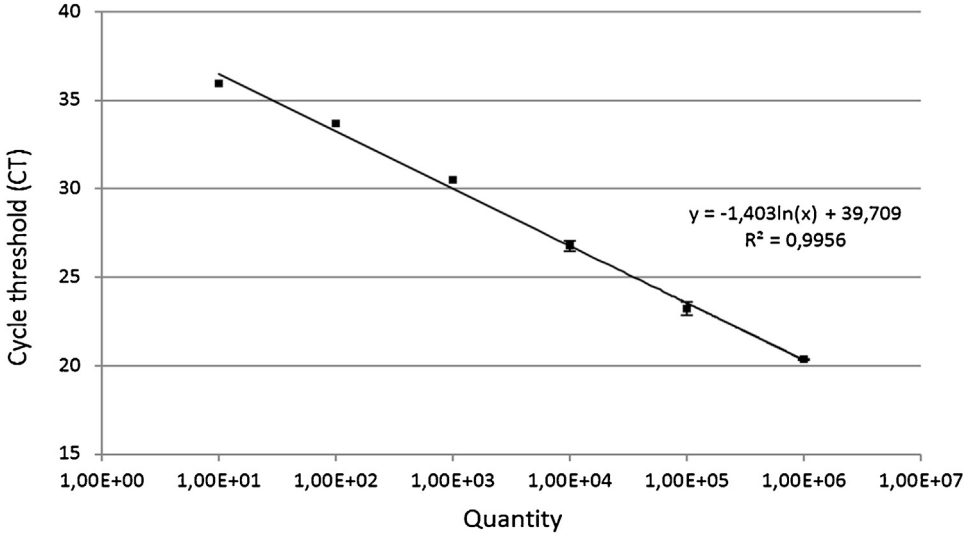


Fig. 2. Standard curve of real-time PCR to detect pathogenic *Leptospira* spp. Determination of the detection threshold by titration of plasmids contaning *lipL32* sequence. The detection limit was 10 DNA molecules with 103.42% efficiency.

Table 2
Bat species and frequencies of real-time PCR results for pathogenic *Leptospira* spp.

Family	Bat species	Feeding habits	No. collected	No. positives	Overall positivity frequency (%)	Positivity frequency within species (%)	Positivity frequency within family (%)
Vespertilionidae	*Eptesicus diminutus*	Insectivorous	3	2	2.2	66.7	75.0
	Eptesicus furinalis		1	0	0.0	0.0	
	Histiotus velatus		2	2	2.2	100.0	
	Lasiurus ega		1	1	1.1	100.0	
	Myotis nigricans		1	1	1.1	100.0	
Molossidae	*Eumops auripendulus*	Insectivorous	5	2	2.2	40.0	35.8
	Eumops patagonicus		3	0	0.0	0.0	
	Molossus molossus		19	4	4.4	21.1	
	Molossus rufus		8	2	2.2	25.0	
	Tadarida brasiliensis		46	21	22.8	45.7	
Phyllostomidae	*Glossophaga soricina*	Nectarivorous	2	1	1.1	50.0	33.3
	Sturnira lilium	Frugivorous	1	0	0.0	0.0	
		Total	**92**	**36**	**39.1**		

negative results for *Tadarida brasiliensis*, the species presenting a higher number of analyzed specimens; and a lower rate of positive results for *Molossus molossus*, the second species with more samples. Considering the family, there seems to be higher frequency of positive results within Vespertilionidae family, although there was no statistical significance; thus, further studies are needed to confirm that different bat families have different degrees of susceptibility to *Leptospira* spp. infection. The leptospiral host specificity could not be accessed, since there was failure to amplify the *secY* gene fragment. This may have been occurred due to low bacterial loads or poor DNA quality, as the bats arrived in the laboratory days after death. Previous studies also report difficulty to amplify larger amplicons from kidney samples positive for leptospiral real-time PCR, even though the bat specimens were captured avoiding DNA quality issues [27,28].

Regarding the feed behavior, the presence of leptospiral DNA was detected in insectivorous species, which refutes the hypothesis that sharing food with rodents increases the likelihood of bats to carry *Leptospira* spp. [29]. As the bats' habitats were not accessed, it was not possible to determine the routes by which the animals were probably infected. Taking into account the *Leptospira* spp. transmission routes, the contact with contaminated soil or water could be considered.

No statistical difference was observed regarding *Leptospira* spp. positivity frequency when analyzing sex, age, local or season of collection. Some of these variables have been described to influence leptospiral carriage in rodents [30]; however, the observed results may be related to low sample number and the way of selection, which was by convenience. The majority of bats evaluated in the present study were found distant from large urban centers and many of them in unusual situations, such as in daylight, sick, or dead. This can indicate increased risk of co-infection with other infectious agents; however, to test this hypothesis, new studies with animals randomly selected should be performed.

Currently, urban sprawl has been responsible for the closer contact of humans and wild animals. Bats often live at roofs, gaps between buildings and urban vegetation, which may have impact on disease transmission to humans and domesticated animals. As the number of analyzed specimens by municipality varied and in general was low, it was not possible to associate bat leptospiral carriage and human cases of leptospirosis; moreover, it was not possible to access the leptospiral serovars infecting the bats. These limitations precluded an analysis about the role of these animals on human leptospirosis transmission, which needs to be further investigated.

There are differences associated with social, health and environmental conditions regarding infectious diseases transmissibility, which are still of major concerns worldwide. Since in the last decades some important infectious diseases have emerged or re-emerged, challenging the global public health security, a better knowledge on the ecology of different infectious agents becomes important. The present study, even though presenting limitations due to lack of *Leptospira* spp. isolation and genotyping, brings new knowledge on leptospiral carriage in Brazilian bats. The data reinforces the need for surveillance of infectious agents, especially the zoonotic ones which are hosted by wild animals.

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