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Leptospira interrogans in bats in Rio Grande do Sul State, Brazil: epidemiologic aspects and phylogeny

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Abstract

Leptospirosis is an infectious disease caused by *Leptospira* spp. and affects animals and humans. Reports of leptospirosis in bats have increased and prompted epidemiological research in Brazil. This study aimed to perform a molecular and epidemiological investigation of pathogenic *Leptospira* spp. in bat kidneys. The total DNA was extracted from 102 kidney samples from chiropterous of different species and cities in Rio Grande do Sul State (RS), Brazil. The polymerase chain reaction was used to amplify a fragment corresponding to *lipL32* gene, which is only present in pathogenic *Leptospira* spp. *lipL32* gene was detected in 22.5% (23/102) of the bat kidney tissues. Phylogenetic analysis showed that *L. interrogans* is circulating in bats in RS. Most species of the bats collected were insectivores. Pathogenic *Leptospira* spp. detection in bats demonstrated that these animals participate in the infection chain of leptospirosis and, therefore, may play as reservoirs and disseminators of this microorganism. Thus, it is important to monitor infectious agents, especially with zoonotic potential in bats.

Keywords Leptospirosis · Chiroptera · Public health · Zoonosis · Molecular epidemiology

Introduction

Leptospirosis is a zoonotic disease caused by bacteria of the genus *Leptospira*; this disease represents significant social, economic, and health impacts in several countries [1–4], especially in tropical and subtropical regions and areas with high humidity and temperatures [3–5]. This bacterium infects various species of domestic and wild animals and humans [6, 7]. Moreover, the genus *Leptospira* is divided into 35 species classified into three phylogenetic groups, which presumably correlate with the bacterium's virulence: saprophytic, intermediate, and pathogenic [8]. Saprophytic bacteria are considered free-living and generally do not cause diseases [9]; intermediate species share a near common ancestor with pathogen species while exhibiting moderate pathogenicity in humans and animals [10, 11], while pathogenic bacteria can cause infection in several animal species and humans, more notably *Leptospira interrogans* [8].

The maintenance of leptospirosis foci in endemic regions is due to the plethora of reservoir hosts that can harbor the bacteria for long periods, which may or may not present clinical signs and quickly spread the infectious agent in the environment and to susceptible species [1]. Transmission of *Leptospira* spp. occurs mainly through contact with urine

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from infected animals or in contaminated environments (soil, mud, or water) via mucous membranes or the skin [12–14].

Rodents constitute the main reservoirs of the etiologic agent [12, 15]; however, there is an increasing role of different wild animal species in the disease cycle. This may be relevant due to the considerable number of wild animal species and contact with domestic animals and humans [16–18]. As such, in the context of zoonotic diseases (e.g., leptospirosis), the role of wild animals in disease epidemiology is crucial, as these animals often coexist with humans, hence their peridomestic habits [19–23]. The relevance of bats as reservoirs of zoonotic pathogens is due to their high mobility, wide distribution, and social behavior [14, 24]. Additionally, these chiropterans constitute one of the most diverse and abundant mammal species groups in neotropical ecosystems [25].

Among the main factors that have favored the increase of contact between wild animals and domestic animals and humans, thereby propitiating the transmission of important zoonoses, we highlight the increased expansion of urban areas and occupation of peri-urban regions, greater population density, global travel, wildlife transit, human encroachment into areas inhabited by wild animals, and expansion and intensification of animal production in natural areas [26–28]. In this context, leptospirosis has been widely researched, and the etiologic agent has been detected in virtually all countries, demonstrating the distribution of the bacterium among different animal species worldwide [29]. Identifying potential wild reservoirs is relevant within the eco-epidemiological context of the diseases [30]. These animals may play an important role in the transmission and dissemination of *Leptospira* spp. under the conditions of reservoirs, infected, symptomatic, or asymptomatic carriers [29]. In this scenario, many wild animals, including bats, are considered reservoirs and possible transmitters of leptospirosis [27, 31, 32].

Infection caused by *Leptospira* spp. occurs by the entry of the agent into the mucous membranes or skin, followed by its multiplication in the blood during the acute phase of the disease or leptospiremia. In animals that develop the condition to survive the acute phase of leptospirosis, the microorganisms migrate to the renal system, lodging in the renal tubules, which may cause them to excrete *Leptospira* spp. in the urine for days or even months [29, 33].

In this context, given the possible role of bats as carriers of the agent and their relationship with human leptospirosis, the sharing of habitats between humans and bats due to urbanization may increase the risk of *Leptospira* spp. transmission by these animals [8, 34]. Chiroptera has been implicated in epidemiological cycles of several emerging and re-emerging zoonoses, such as rabies [35, 36], severe acute respiratory syndrome (SARS) [37], and Ebola [38]. In addition, despite the origin of the etiologic agent of the COVID-19 pandemic not being well defined, bats of the genus *Rhinolophus* have been listed as the likely origin of the novel coronavirus, SARS-CoV-2, the causative agent of COVID-19 [39–42].

Molecular detection studies of *Leptospira* spp. in wild and synanthropic animals are necessary to demonstrate epidemiological aspects of leptospirosis in a given region since they can act as reservoirs for the bacteria and transmit it to other animal species (domestic and wild) and humans [13, 43–45]. Given this context, we aimed to perform an epidemiological and molecular investigation of pathogenic *Leptospira* spp. in bats collected in Rio Grande do Sul State (RS), Brazil.

Materials and methods

This study analyzed kidney tissues from 102 bats (204 kidneys) collected in different urban areas of RS, Brazil, from 2016 to 2021. The samples were obtained by convenience sampling, since the dead bats came from different municipalities and were sent to the Centro Estadual de Vigilância em Saúde (CEVS) in Porto Alegre (RS) for rabies diagnosis. All animals analyzed in this study tested negative for rabies. Subsequently, the chiroptera were kept frozen and sent to the Universidade Federal de Santa Maria (UFSM) for analysis. The bats were taxonomically identified according to their family, genus, and species according to Díaz et al. [46] (Table 1). Subsequently, the animals were sexed, weighed, kidney tissue fragments aseptically collected (~20 mg), conditioned in Eppendorf polypropylene microtubes, and kept at − 20 °C until molecular analyses.

Total DNA extraction was performed according to the protocol described by Botton et al. with modifications adapted for tissues [47]. The fragments of the kidney tissue samples were macerated in lysis buffer containing 2-βmercaptoethanol, 2% sodium dodecyl sulfate (SDS), and 10% cetyltrimethylammonium bromide (CTAB), and 5 N Sodium chloride(NaCl) was added. Extraction was then performed with phenol and chloroform, and the total DNA was resuspended in 30 uL of sterile Tris–EDTA (TE) buffer. In the end, the DNA was quantified in a NanoDrop® spectrophotometer, and the polymerase chain reaction (PCR) was performed to amplify a fragment of *lipL32* gene, which encodes external membrane proteins that are exclusively present in pathogenic *Leptospira* spp.

The sensitivity of the test was measured by the detection threshold of the positive control using the same primers (1.5×10^3 *Leptospira interrogans* cells corresponding to 8,3 ng/µL), and a sample PCR was prepared for a final volume of 12.5 µL containing 1 × buffer (Ludwig Biotec®, Brazil), 1.5 mM $MgCl_2$ (Ludwig Biotec®, Brazil), 0.2 mM dNTPs (Ludwig Biotec®, Brazil), 2.5 U of Taq

Table 1 Frequency of molecular detection of *Leptospira* spp. pathogens in bats collected from 2016 to 2021 in Rio Grande do Sul State, Brazil

Family	Bat species	Feeding habits	Bats collected (N)	Positive DNA (N/%)	Positivity per species (%)	Positivity per family (%)
Molossidae	*Tadarida brasiliensis*	Insectivores	50	13/56.6	26.0	21.4
	Molossus molossus		14	2/8.7	14.3	
	Molossus correntium		11	3/13.1	27.3	
	Molussus rufus		8	0/0	0.0	
	Nyctinomops laticaudatus		1	0/0	0.0	
Vespertilionidae	*Histiotus velatus*	Insectivores	1	1/4.3	100.0	60.0
	Eptesicus furinalis		1	0/0	0.0	
	Myotis levis		1	1/4.3	100.0	
	Eptesicus brasiliensis		1	0/0	0.0	
	Lasiurus blossevillii		1	1/4.3	100.0	
Phyllostomidae	*Sturnira lilium*	Frugivores	1	0/0	0.0	0.0
Unidentified samples			12	2/8.7		
TOTAL			**102**	**23**		

DNA polymerase (Ludwig Biotec®, Brazil), 0.5 µM of each primer (Invitrogen®, Brazil) LipL32-45F (5′-AAG CAT TACCGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′) [48], and 2.5 µL (330 ng/µL) of the extracted DNA sample. Amplification was performed in a PCR thermal cycler (K960, TION96) consisting of an initial denaturation of 94 °C/2 min, 35 cycles of 94 °C/30 s, 53 °C/30 s, 72 °C/1 min, followed by a final extension at 72 °C/5 min and 4 °C/∞. The PCR products were analyzed in horizontal 1% agarose gel electrophoresis stained with Gel Red®(Kasvi), observed under ultraviolet light, and photo-documented.

Sequencing analyses later identified positive samples of *Leptospira* (*lipL32*) in PCR. PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and sequenced. The sequences obtained were aligned with the MEGA X [49] software and compared with each other and the reference sequences available in the GenBank. The phylogenetic tree [50] was constructed with Bayesian Analysis [51], using the bootstrap was resampled as a test of phylogeny using 500 replications [52].

Results

The number of bats analyzed per municipality is shown in Table 2. The bats were classified into eleven species of the families Phyllostomidae, Molossidae, and Vespertilionidae. The distribution of bats by family, species, and feeding habits is listed in Table 1. Most samples (49.0%; 50/102) were free-tailed bats (*Tadarida brasiliensis*). Regarding the animals' sex, 60.8% (62/102) were male, and 39.2% (40/102) were female. The PCR revealed that the amplification of a fragment of 242 base pairs (bp) corresponds to the expected size for the *lipL32* gene in 23 (22.5%) samples, which is considered positive for pathogenic *Leptospira* spp. The sensitivity of the test was detection up to 1.5×10^3 bacteria/ml. Among the amplified samples, six were identified in at least one different bat species evaluated (Table 1). Among the males, the expected DNA fragment was detected in 52.2% (12/23) and among females in 47.8% (11/23).

Among the species with the highest number of specimens analyzed, *Tadarida brasiliensis* showed 56.6% (13/23) of animals positive for pathogenic *Leptospira* spp. *Molossus correntium* and *Molossus molossus* showed positivity rates of 13.1% (3/23) and 8.7% (2/23), respectively. In the other bat species analyzed, the infection rate detected was 4.1% for *Histiotus velatus* (1/23), *Myotis levis* (1/23), and *Lasiurus blossevillii* (1/23). It was not possible to identify the presence of DNA in samples from individuals of the species *Molossus rufus*, *Nyctinomops laticaudatus*, *Eptesicus furinalis*, *Eptesicus brasiliensis*, and *Sturnira lilium*.

The samples were from 31 municipalities distributed in the seven mesoregions of RS. It was possible to observe that 10 cities had at least one positive bat (Table 2).

Based on the phylogenetic analyses (Fig. 1), we found *lipL32* gene fragments from *Leptospira* spp. detected in bats in Rio Grande do Sul State were clustered in *L. interrogans* pathogenic group. Comparing the analyzed samples, was obtained 100% identity with *L. interrogans* from the sequences available on the GenBank (MT482312 and KM211316), originating from samples of bats (*Myotis myotis*) and swine (*Sus scrofa domesticus*), respectively.

Table 2 Geographical distribution of kidney tissue samples of bats positive for pathogenic *Leptospira* spp. from different cities of Rio Grande do Sul State, Brazil

City	Collected samples	DNA positive samples
Pelotas	21	1
Canoas	13	5
Rio Grande	11	5
Porto Alegre	11	2
Caxias do Sul	7	4
Santa Maria	5	-
Ijuí	3	1
São Leopoldo	3	-
Bento Gonçalves	2	-
Campo Bom	2	-
Agudo	1	-
Alegrete	1	-
Cachoerinha	1	-
Camaquã	1	-
Capão do leão	1	1
Capivari do Sul	1	1
Eldorado do Sul	1	-
Gramado	1	-
Gravataí	1	-
Guaíba	1	-
Humaitá	1	1
Igrejinha	1	-
Ivoti	1	-
Nova Alvorada	1	-
Nova Boa Vista	1	-
Passo Fundo	1	-
São Gabriel	1	-
Sapucaia do Sul	1	-
Sertão Santana	1	1
Taquari	1	-
Tiradentes do Sul	1	-
Toropi	1	-
Unknown origin	2	1
Total	**102**	**23**

-: DNA sample was not detected by molecular analysis

Discussion

In Brazil, a frequency of 39.1% (36/92) was observed in RS and Santa Catarina States [18], 1.8% (6/343) in São Paulo State [53], and 7.8% (16/204) in Botucatu [54]. Nevertheless, in this study, it was possible to detect the presence of DNA of pathogenic *Leptospira* spp. in 22.5% (23/102) of bat kidney tissues from different RS regions. The positive samples were from metropolitan, northeastern, northwestern, and southeastern Rio Grande do Sul, which have higher temperatures, humidity, and high annual rainfall distribution than other Brazilian regions [55].

Here, the presence of pathogenic *Leptospira* spp. DNA was detected primarily in insectivorous bat species. Phylogenetic analysis revealed that *L. interrogans* circulates among chiropteran bats in southern Brazil, an important region of international transit of people and animals circulating in the country, Uruguay, and Argentina. Notably, this is the first known study using phylogenetic analysis to detect *Leptospira interrrogans* in bats in Brazil, as previous studies [53, 54] employed serological analysis and only one study [18] performed analysis by PCR.

In this study, there were no differences in the sex of the animals: 52.2% (12/23) were males and 47.8% (11/23) were females. Nonetheless, Wilkinson [56] described that bat colonies have the habit of licking other bats and females perform regurgitation to feed their offspring. This behavior favors the proximity of animals, consequently increasing the likelihood of transmitting pathogens, including *Leptospira* spp.

The presence of pathogenic *Leptospira* spp. DNA has been detected primarily in insectivorous bat species. Harkin et al. [57] hypothesized that a possible mode of transmission of *Leptospira* spp. for bats would be sharing food with rodents. However, because the habitats of the bats could not be assessed, it was impossible to determine the routes by which the animals were likely infected. In urban areas, bats usually nest in ceiling panels, which are also places that host other animal species such as rodents, lacertids, columbids, and even marsupial species [58]. Thus, proximity between the species may increase the risk of contact with secretions and/or excretions and the contamination of the agent in utensils and food consumed by humans and domestic animals. Hence, a network of possibilities for transmission of the agent emerges in the multiple interactions between various mammalian species, including humans [40].

Rodents and bats share roosting sites in peri-urban and rural areas, such as sheds where food and grain are stored and animals are raised. It is common to find nests and bats hanging from farm roofs and structures, whose waste, such as urine, falls on the animals and their food [40]. In an environment with high habitat overlap, shared resources among species bring individuals closer together and intensify the possibility of spreading *Leptospira* spp. [19].

In this study, all bats positive for *Leptospira* spp. were insectivores. These animals possibly have characteristics of synanthropy since these bat species came from urban environments [59]. Urban afforestation is a potential source of food and shelter for insects and insectivorous bats. In addition, public lighting attracts insects around the light beams, predisposing the joint occurrence of insectivorous bats [60, 61].

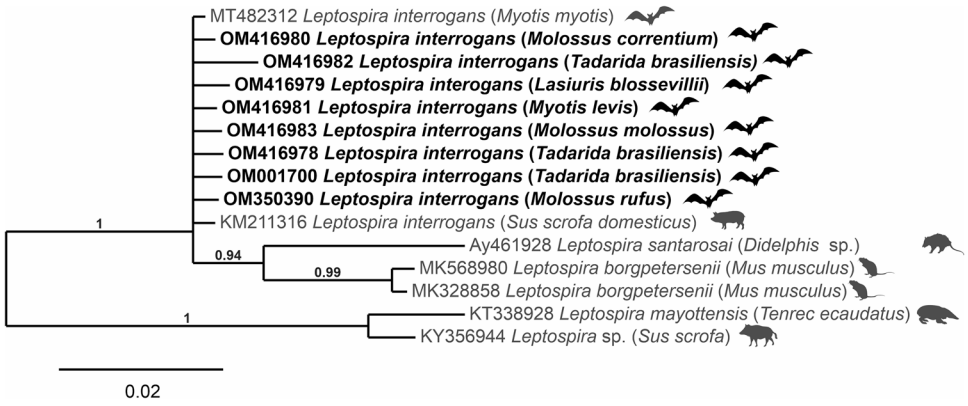

Fig. 1 Phylogenetic analysis of *Leptospira interrogans lipL32* gene sequences obtained of bats from Southern Brazil. The analysis was performed by Bayesian method, with 500 bootstraps, in MEGA X software. *Leptospira interrogans* sequences of the analyzed samples are written in bold

The region with a higher concentration of bats that tested positive for pathogenic *Leptospira* spp. corresponds to the metropolitan area of Porto Alegre (capital of RS). In this area, there is greater urbanization, with a more concentration of population and a large industrial and commercial area [62]. In addition, this region has degraded environmental areas with accumulations of waste and water (water springs, water reservoirs such as public and private pools, and water tanks) [55, 62, 63], favoring the contact of synanthropic animals, especially rodents, and stray animals. These environmental conditions contribute epidemiologically to the development of *Leptospira* spp. in these places, thereby making possible the favoring the transmission of the agent to insectivorous bats.

The current incidence of leptospirosis in human and animal is unknown due to the lack of information [64], large proportion of subclinical infection and non-specific course, and unavailable diagnostic methods in laboratories public and private health services, thus impairing detection [65]. In Porto Alegre, leptospirosis incidence from 1996 to 2007 varied between 0.85/100 thousand inhabitants (2004) and 7.14/100 thousand inhabitants (2001), and this was associated with the disorderly growth of the city, lack of environmental sanitation and public water supply, domestic sewage canalization, and waste management, increasing the problem of synanthropic rodent infestation [66]. The southern region of RS, another highly relevant area due to the positive results of our study, is also indicated as a place of high leptospirosis rates [64].

Leptospira spp. have been detected in roughly 50 bat species belonging to 8 families in tropical and subtropical regions of the planet [9]. In several countries of the Americas, the presence of pathogenic *Leptospira* spp. DNA has been detected in bats. In Mexico, DNA detection of *L. noguchii* and *L. weilii* in bats has been reported [67], the first country to report pathogenic *Leptospira* spp. species in flying mammals in North America. Reports in Peru have addressed DNA detection of *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, and the species of intermediate pathogenicity such as *L. fainei* [17, 68]. In the Peruvian Amazon basin, Bunnell et al. [68] found that 35% (7/20) of bat kidneys showed DNA of pathogenic *Leptospira* spp. by PCR. Matthias et al. [17] tested 589 bats from the same area and found only 20 positive kidneys using PCR and three urine samples positive by culture. In Argentina, Ramirez et al. [69] found 20% of DNA from *Leptospira* spp. (14/70) in insectivorous bats. In Colombia, Mateus et al. [14] observed 26.9% of the presence of *Leptospira* spp. (7/26) and 15.4% for pathogenic *Leptospira* (4/26) and Monroy [70], with 9.70% for the presence of DNA from *Leptospira* spp. (20/206). Nonetheless, Harkin et al. [57] did not detect pathogenic *Leptospira* spp. in 98 kidney tissue samples from the US states of Kansas and Nebraska.

On other continents, different studies have detected the presence of *Leptospira* spp. in chiropterans. In Madagascar, Lagadec et al. [71] obtained a total positivity of 35% (18/52); meanwhile, in Comoros, the same researchers observed 12% (9/77). In Tanzania, Mgode et al. [72] found 19% (7/36) of the samples positive. In Zambia (the Republic of Congo), Ogawa et al. [73] obtained 15% (79/529) of positive samples. In Australia, Tulsiani et al. [30] reported 11% (19/173), while the prevalence of *Leptospira* spp. in bats was 56.7% (34/60) in central China; however, in the northern region of this country, this rate was slightly higher, reaching 62% (62/124) [7].

Regarding the participation and importance of species in leptospirosis, some studies can be highlighted, including Han et al. [7], that reported *Myotis* spp. as a species of a high prevalence of *Leptospira* spp., with 53% and 63% in central and the northern region of China, respectivelly. Bats of the genus *Myotis* belong to the family Vespertilionidae, which differs from our results, since we found pathogenic *Leptospira* spp. in bats of the Molossidae family, especially in *Tadarida brasiliensis*. This fact can be explained by the circulation of this species mainly in humid places, where it is believed there is a higher probability of the presence of *Leptospira* spp. [74]. Han et al. [7] also detected pathogenic *Leptospira* spp. in *M. fimbriatus*, *M. ricketti*, and *M. pequinius*, which also live in the wetlands of Mengyin County, China.

Conclusion

The presence of pathogenic *Leptospira* spp. DNA was found in wild bats in different macro-regions of Rio Grande do Sul, southern Brazil. The highest occurrence was observed in insectivorous bats of the species *Tadarida brasiliensis*. This was the first study in Brazil combining a molecular detection and phylogenetic analysis of pathogenic *Leptospira* spp. DNA confirming the presence of *L. interrogans* in bats. Our findings corroborate the elucidation of the epidemiology of leptospirosis in southern Brazil, an important region due to the transit of people and animals among neighboring countries. However, further research is needed on the ecology of this agent in these mammals, reinforcing the need for surveillance of infectious agents, especially zoonotic ones, in wild animals.

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Declarations

Ethics approval No ethical approval was sought or required for this work as it is a theoretical contribution.

Conflict of interest The authors declare no competing interests.

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