

SHORT COMMUNICATION

New records of *Leptospira* spp. in wild marsupials and a rodent in the eastern Brazilian Amazon through PCR detection

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ABSTRACT

We analyzed the presence of *Leptospira* spp. in liver and kidney tissue of wild marsupials and rodents trapped in a periurban forest in the eastern Brazilian Amazon. We examined 25 individuals of four marsupial and seven rodent species for the presence of the 16S rRNA gene of *Leptospira* in the DNA extracted from 47 liver and kidney tissue samples using PCR. We detected positive samples in 12% (3/25) of the individuals, in kidney fragments of two marsupial species (*Didelphis marsupialis* and *Marmosops pinheiroi*) and in a liver fragment of one rodent species (*Echymys chrysurus*). These are the first records of *Leptospira* spp. in *M. pinheiroi* and *E. chrysurus* and it is the first molecular survey of marsupials and rodents in the Brazilian Amazon.

KEYWORDS: Didelphimorphia, Rodentia, live traps, kidney, liver, 16S rRNA

Novos registros de *Leptospira* spp. em marsupiais e um roedor silvestres na Amazônia Oriental por detecção por PCR

RESUMO

Analisamos a presença de *Leptospira* spp. em tecido hepático e renal de marsupiais e roedores silvestres capturados em uma floresta periurbana no leste da Amazônia brasileira. Foram examinados 25 indivíduos de quatro espécies de marsupial e sete de roedor quanto à presença do gene 16S rRNA de *Leptospira* no DNA extraído de 47 amostras de tecido hepático e renal usando PCR. Detectamos amostras positivas em 12% (3/25) dos indivíduos, em fragmentos renais de duas espécies de marsupial (*Didelphis marsupialis* e *Marmosops pinheiroi*) e em um fragmento hepático de uma espécie de roedor (*Echymys chrysurus*). Estes são os primeiros registros de *Leptospira* spp. em *M. pinheiroi* e *E. chrysurus*, e é o primeiro levantamento molecular de marsupiais e roedores na Amazônia brasileira.

PALAVRAS-CHAVE: Didelphimorphia, Rodentia, armadilhas, rim, fígado, 16S rRNA

Leptospirosis is a globally distributed zoonosis and is known to have domestic and wild animal species as reservoirs, mainly small mammals such as rodents and marsupials (Adler and De La Peña Moctezuma 2010). In the Brazilian Amazon, the intensification of land demand generates the emergence and expansion of urban borders, contributing to the increase in deforestation (Ribeiro *et al.* 2018). Deforestation generates environmental changes and ecological disturbances that facilitate contact with wild species, favoring the incidence of

diseases transmitted by animals (Saccaro Junior *et al.* 2015). Serological surveys have also suggested that these animals might be potential reservoirs of *Leptospira* spp. (Valbuena-Torrealba and Péfaur-Veja 2015; Paixão *et al.* 2014; Silva *et al.* 2013; Ruiz-Piña *et al.* 2002).

In the Amazon region, few studies have investigated the presence of *Leptospira* in marsupials and rodents. Burnnel *et al.* (2000) identified DNA from *Leptospira* spp. in kidney

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samples from 20% of rodents and 39% of marsupials sampled in Iquitos, in the Peruvian Amazon, and identified DNA from pathogenic leptospire in *Philander* sp., *Marmosops bishopi* (Pine, 1981) and *Marmosops noctivagus* (Tschudi, 1845), suggesting that marsupials are the most significant hosts for the potential transmission of pathogenic leptospire to humans in the region.

There are few data on the infection of wild species by leptospire in the Brazilian Amazon. Lins and Lopes (1984) identified isolates of serovar Ballum in bacterial cultures of renal tissue of two rodents (*Proechimys* sp.) and a marsupial (*Didelphis marsupialis* Linnaeus, 1758), and two unidentified isolates in rodents and four in marsupials. Mesquita *et al.* (2018) reported a prevalence of 36.8% of seropositive *D. marsupialis*, *Caluromys* sp. and *Marmosa murina* (Linnaeus, 1758) for antibodies to serovars Icterohaemorrhagiae, Panama and Nupezo. Here, we report the first data on detection of *Leptospira* spp. in wild marsupials and rodents from the state of Pará using PCR.

Small mammal trapping was carried out in a secondary forest fragment located in the Expedito Ribeiro settlement (1°17'06.76"S, 48°15'44.04"W), in the municipality of Santa Bárbara, Pará state, in the eastern Brazilian Amazon (Figure 1). The climate in the region is hot and humid, with a relative air humidity of approximately 85%, with average temperature around 26 °C throughout the year and annual rainfall exceeding 2,550 mm, with more frequent rains from January to June (Santos and Jardim 2006).

Animals were captured using baited Sherman, Tomahawk and pitfall traps during at least 10 consecutive nights in two field expeditions in October 2015 and April 2016. Captured marsupials and rodents were first anesthetized and then euthanized as recommended by the National Council for Animal Experimentation Control (CONCEA). Small mammal sampling was authorized by Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis – IBAMA (37174-1) and the ethics committee on animal use of the Evandro Chagas Institute – CEUA/IEC (protocol 028/2014).

Pregnant or lactating females and young were excluded and released at the place of capture. Euthanized animals were necropsied *in locu* to collect fragments of liver and kidney. The tissue samples were stored in 1.5 mL sterile microtubes in a nitrogen canister for transport to the Zoonosis and Public Health Laboratory of the Federal University of Pará (UFPA). The species were identified based on morphometric measurements and cranial analysis by expert taxonomists from Universidade Federal do Pará - UFPA and Museu Paraense Emílio Goeldi.

The extraction of DNA from the liver and kidney samples was performed with the Illustra™ Tissue and Cell Prep Mini Spin kit (GE Healthcare®). DNA was detected using the primers described by Mérien *et al.* (1992), (5'GGCGGCGGTCTTAAACATG3') and (5'TTCCCCCATTGAGCCAAGATT3'), which amplify 331 base pairs of the *Leptospira* spp. 16S rRNA gene. Amplification was performed by polymerase chain reaction

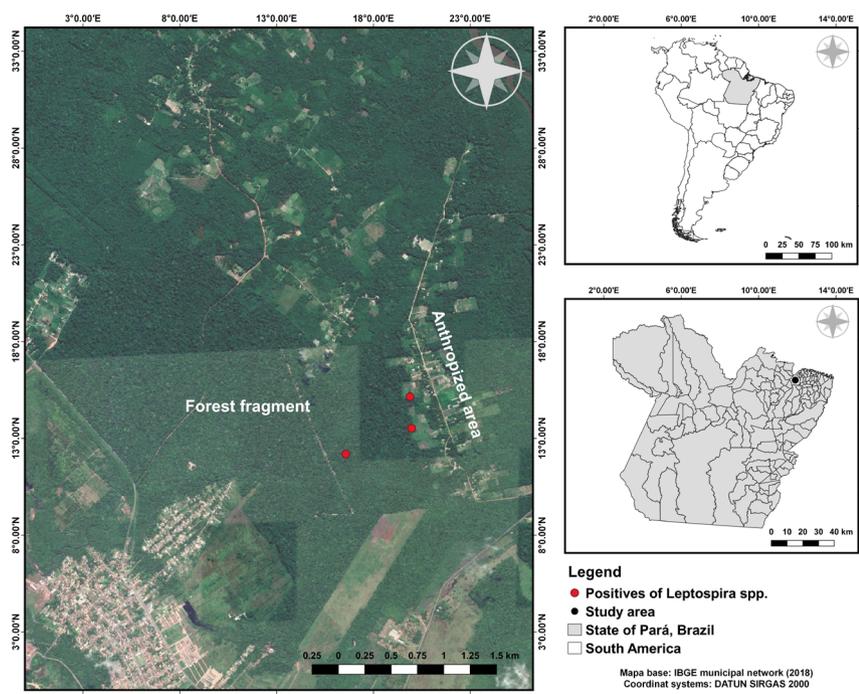


Figure 1. Location of Pará state in Brazil and South America, and of the study area within Pará state. The satellite image shows the sampled forest fragment and nearby urban areas. Red circles indicate the capture site of the rodent and marsupials positive for *Leptospira* spp. This figure is in color in the electronic version.

(PCR). The PCR mixture contained 2.5 µL buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.0), 1.0 µL MgCl₂ (1.5 mM), 1.0 µL dNTP solution (1.0 mM), 0.3 µL Taq DNA polymerase (Ludwig[®]), 1 µL of each oligonucleotide primer (2.5 pmol), 5 µL DNA template, and 13.2 µL ultrapure water in a final volume of 25 µL.

The amplification reactions were each carried out with a positive control of 25 ng µl⁻¹ DNA from *Leptospira interrogans* serovar canicola and a negative control from a tissue sample of the rodent species *Rattus norvegicus* (Berkenhout, 1769) free of *Leptospira* from an authorized vivarium, and ultrapure nuclease-free water was used as a contamination control.

The thermal cycling steps in PCR included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1.5 min, and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. All amplification reactions were performed in a Veriti[®] 96-Well Thermal Cycler (Applied Biosystems). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel; the gel was stained with a safe dye (KASVI) and visualized under ultraviolet light on a transilluminator coupled to a photo-documentation system (Gel Doc[™] XR+ Imaging System, BioRad).

We analyzed 47 liver and kidney samples from 11 marsupials belonging to four species and 14 rodents belonging to seven species, including a synanthropic rodent (*Rattus rattus*), suggesting that these animals typically from urban surroundings come into contact with wild animals in the study area, which may pose a risk of agent transmission (Table 1).

Leptospira spp. DNA was detected in 12% (3/25) of the individuals. The rate of infection was higher in the marsupials, with 18.2% (2/11) infected animals as compared to the rodents, with 7.2% (1/14) infected animal (Table 1). *Leptospira* spp. DNA was present in the kidney of one individual each of *Didelphis marsupialis* and *Marmosops pinheiroi* (Pine, 1981) (Didelphidae) captured on the edge of the forest fragment, and the liver of the only individual of *Echimyus chrysurus* (Zimmermann, 1780) (Echimyidae) captured inside the forest fragment (Table 1; Figure 1).

This is the first report of detection of *Leptospira* DNA in marsupials in the Brazilian Amazon, which agrees with the findings of Burnell *et al.* (2000), who detected *Leptospira* spp. DNA in kidney samples of marsupials from the Peruvian Amazon using G1/G2 primers and identified infection by pathogenic leptospires through the 23S rRNA gene in three marsupial species (*Philander* sp., *Marmosops bishopi* and *Marmosops noctivagus*). *Didelphis marsupialis* is the marsupial most frequently associated with *Leptospira*. The species was tested positive for serovar ballum isolates by Lins and Lopes (1984) in the Brazilian Amazon, serovars szwajizak and icterohaemorrhagiae by Rosa *et al.* (1975) in southeastern Brazil, and serovars Djasiman, Sejroe and Cynopteri by

Hidalgo and Sulzer (1984) in Peru. To the best of our knowledge, this is the first report of infection by *Leptospira* in *Marmosops pinheiroi*.

Antibodies against *Leptospira* spp. had already been detected in *D. marsupialis*, *Caluromys* sp. and *Marmosa murina* in the same region in the state of Pará (Mesquita *et al.* 2018), which, together with our detection of *Leptospira* DNA in kidney samples from *D. marsupialis* and *M. pinheiroi*, suggests that marsupials are potential reservoirs of the agent at the region.

This is also the first report of infection by *Leptospira* spp. in *Echimyus chrysurus*. Vieira *et al.* (2018) reported an average infection rate of 20% among several species of wild rodents in Latin America, not including the species identified as positive in the present study, suggesting that more studies are necessary to evaluate the role of wild rodents as a reservoir of *Leptospira*. Bacterial DNA in rodent blood and kidney samples has also been found in the Mata Atlântica biome in Brazil (Paixão *et al.* 2014; Vieira *et al.* 2019) 13 rats were captured at seven locations of the Centre for the Conservation of Wild Fauna (CCWF). *Leptospira* DNA was reported in 6% of wild small mammals captured in three forest areas in Germany (Obiegala *et al.* 2016), in 20.4% of rodents captured in urban and rural areas in Chile (Muñoz-Zanzi *et al.* 2014) and in 13% of wild rodents captured in Malaysia (Latifah *et al.* 2012).

Unfortunately, our small sample size does not allow for reliable inferences regarding prevalence of *Leptospira* spp. in the community of arboreal mammals in the study area. The low number of animals captured was probably due to the abundance of food in the forest, which made the bait in the

Table 1. Species of marsupial and rodent captured in a forest fragment in the municipality of Santa Bárbara, Pará state (Brazilian Amazon) for detection of *Leptospira* spp. DNA in tissue samples. N = number of individuals captured. Prevalence is indicated for positive cases.

Sampled species	N	Prevalence
Rodentia		
<i>Oecomys</i> cf. <i>bicolor</i>	3	Negative
<i>Oecomys</i> <i>paricola</i>	6	Negative
<i>Guerlinguetus</i> cf. <i>gilvicularis</i>	1	Negative
<i>Echimyus chrysurus</i>	1	01/01 (100%)
<i>Neacomys</i> sp.	1	Negative
<i>Nectomys</i> cf. <i>rattus</i>	1	Negative
<i>Rattus rattus</i>	1	Negative
Didelphimorphia		
<i>Marmosops pinheiroi</i>	6	01/06 (16.4%)
<i>Didelphis marsupialis</i>	3	01/03 (33.3%)
<i>Philander opossum</i>	1	Negative
<i>Metachirus nudicaudatus</i>	1	Negative
Total	25	3/25 (12%)

traps less attractive. In addition, the concentration of DNA amplified from the PCR-positive samples was not sufficient for sequencing and, therefore, the *Leptospira* species infecting the animals could not be identified. Further research should confirm whether marsupials and rodents in the region carry pathogenic forms of *Leptospira* and determine the prevalence and circulation dynamics of this pathogen among wildlife in the region.

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