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Review Article

Leptospira spp. in Opossums *Didelphis aurita* from the Atlantic Forest, Rio de Janeiro, Brazil

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Abstract

Leptospirosis is a worldwide zoonosis which has been recently recognized as a paradigm to the One Health approach due to the interface of human-animal-environment observed in the transmission cycles. A total of 40 opossums identified as *Didelphis aurita* were captured at the Campus FIOCRUZ within the Atlantic Forest, Rio de Janeiro, Brazil to evaluate their possible role as carriers of *Leptospira* spp. The 40 serum samples were submitted to the microagglutination test using a panel of 19 reference strains. Kidney fragments of 13 animals out of 40 were used to perform PCR and standard procedures to isolate leptospires in culture. The percentage of positive sera was 10% (4 out of 40). The PCR showed 4 positive kidney samples out of 13 (31%). Two strains were isolated in culture medium (15.4%). Multilocus sequence typing (MLST) analysis of both isolates did not show a 100% match with any other sequence types deposited at the database used (http://pubmlst.org/leptospira/). The closest match of one isolate was with ST 177 represented by one strain of *L. santarosai* and the closest match of the other isolate was with STs 166 and 171 represented by strains of *L. noguchii*. It is the first report indicating the potential of opossums *Didelphis aurita* as a carrier of *Leptospira* spp.

INTRODUCTION

The human-animal-environment interface is the central issue in the mechanisms of transmission of leptospirosis and consequently concerning the occurrence of infection among animals in different areas of Latin America [1]. Geographic and climatic factors are determinant regarding the existence of sporadic cases or epidemic outbreaks since water and moist soil are the primary vehicles in the indirect transmission of leptospires from animal carriers to human beings. Heavy rains and floods are often associated with epidemics outbreaks of leptospirosis, which are frequently caused by weather changes or as a consequence of climate change [2].

Leptospirosis in humans is relatively underestimated but causes impact due to the high morbidity and fatality rates. Based on a systematic review of 80 published morbidity and mortality studies and databases from 34 countries, it is estimated that there are 1,03 million cases and 58,900 deaths each year. These estimates place leptospirosis as a leading zoonotic cause of morbidity and mortality [3]. Official data from Brazil's surveillance system shows that in the period 2010-2015 a total of 24,057 cases and 2,023 deaths were recorded. The disease is on the list of priorities for the country's surveillance system [2,4].

Concerns about animal leptospirosis are related to animal health and economic loses. There is no available estimation the global level or in Brazil, but official data of the OIE shows a broad distribution of epidemics in Latin America (LA) [1,2]. The multiple risk factors involving the interface human-animal-environment represent a big challenge for surveillance and control measures. Concerning the risks related to the circulation of leptospires between wild animals little is known regarding animal species, geographical distribution and impact, although the relative risk is widely recognized in the scientific literature from sporadic studies over time and sparse in different geographic areas [1].

The biodiversity of *Leptospira* and the diversity of animal species involved in the transmission of the bacteria should be highlighted as the main challenge for scientific research and possible interventions in public and animal health [1,2]. The genus comprises 22 species grouped in pathogenic, intermediate and saprophytic species. Currently, there are more than 250 named, potentially pathogenic serovars according to the current criteria for the taxonomy of the genus [5].This exploratory study focuses on a common possum species in the Atlantic Forest of Rio de Janeiro, Brazil. The geographic distribution of the species extends from East to West of Brazil, reaching Paraguay and Argentina. It is a common species in its area of distribution with adaptation to variable habitats. They usually live near waterways. They are found in an average population density of 1.4 individuals/ha. They typically approach of houses in transition areas between forests and urban areas [6].

MATERIALS AND METHODS

Area of study

The opossums were captured at Campus FIOCRUZ of Atlantic Forest (CFMA, acronym in Portuguese), which corresponds to an area of 5 million square meters located in Maciço da PedraBranca, Rio de Janeiro, Brazil. The area of environmental preservation is equivalent to 50% of the total. In the surroundings, there is substantial degradation due to growing human occupation. It is an area of expansion of FIOCRUZ with projects that aim at the sustainable and healthy balance between human occupation and preservation of the environment giving support to public policies in the area of health and environment.

Animal trapping and handling

Animal trapping and handling were carried out according to previously described methodology [6]. The project was licensed by the institutional ethics committee for the use of animals with research purposes (LW-81/12 license). The captures were made with the authorization of the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA, acronym in Portuguese), license no. 13373-1. In brief, the trapping was carried out during one year. The animals were identified so that they would be recognized if they were caught more than once. Each sampling process was carried out in four expeditions lasting 5 nights. Three environments were sampled inside the Campus: wild, transitional and peri-urban areas. Only adult animals were used, excluding pregnant or young offspring. In each of the three areas were established two linear transects with 20 capture stations each, totaling 240 traps with food baits appropriate to the eating habits of local animals. All captured animals were identified at species-level. The opossums of the species *Didelphis aurita* represented the object for this study. Blood and kidney fragments of the animals were obtained and sent to the laboratory for serological procedures, PCR, and isolation of leptospires from renal tissue. The transport was carried out in adequate containers to maintain the ideal conditions for the laboratory procedures and to ensure biosafety requirements.

Serology

The serology of the 40 animals was performed according to previously described procedures, using a battery of 19 reference serovars as recommended for the diagnosis of humans and animals or for the detection of anti-*leptospira* antibodies [7]. All sera were further tested with the two strains isolated from the two opossums. The whole panel of reference strains included the following serovars: Icterohaemorrhagiae, Copenhageni, Canicola, Grippotyphosa, Pomona, Australis, Bataviae, Celledoni, Cynopteri, Djasiman, Hebdomadis, Javanica, Panama, Pyrogenes, Hardjo, Saxkoebing, Shermani, Tarassovi and Patoc.

PCR in renal tissues

For DNA extraction the Dneasy Blood and Tissue Kit (Qiagen) was used according to the manufacturer's specifications using a similar amount of renal fragments. The protocol used follows broadly previous publications. The flaB and lipL41 primers were used [8].

Isolation of *Leptospira*

Kidney fragments from 13 possums were used to isolate leptospiras and search for specific fragments of DNA by PCR. Fragments were extracted from the region between the cortex and medullary region using half of a kidney of each animal. Punch for biopsy of 4 mm were used in this procedure. The fragments were homogenized in 15mL of sterile saline solution, pH 7.2. After a 20 minutes rest period, 0,5mL of the supernatant was inoculated into tubes with 4.5mL of the EMJH semi-solid medium and incubated at a temperature of 28-30^{0}C in the dark.

PCR in renal tissues

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CHARACTERIZATION OF ISOLATED STRAINS

Serological characterization

MAT was carried out with a panel of 17 rabbit antisera against reference serovars, representing the serogroups Icterohaemorrhagiae, Canicola, Grippothyphosa, Pomona, Australis, Ballum, Bataviae, Celledoni, Cynopteri, Djasiman, Hebdomadis, Javanica, Panama, Pyrogenes, Sejroe, Shermani, and Tarassovi. Antisera were obtained from the Royal Tropical Institute (Amsterdam, The Netherlands).

Genotypic characterization by multilocus sequence typing (MLST)

The analysis of internal fragments of 7 house-keeping genes (ptnA, sucA, pfkB, tpiA, mreA, glmU, and caiB) was performed according to protocols previously published [10] Purified amplicons were sequenced at the Platform of DNA Sequencing (core facility) - RPT01A-PDTIS/FIOCRUZ (http://plataformas.cdts.fiocruz.br/). Sequences were aligned with Clustal W and analyzed at the MLST website (http://pubmlst.org/leptospira/).

RESULTS AND DISCUSSION

Serological evidence of infection was found in 10% (4 out of 40) of *Didelphis aurita* sera submitted to MAT. Three reacted only with the serovar Panama in titers from 1: 200, 1: 400 and 1: 800 respectively. One positive serum reacted exclusively with the homologous antigen (a strain isolated from the animal itself) with the titer 1:400 (Table 1). The data indicate evidence of infection and the possibility of the circulation of the serovar Panama in 3 out of 4 positive animals identified as D-13, D-18, and D-30. The findings of a positive reaction only with the homologous strain isolated from the same animal (identified as D-29) suggest the possibility of infection by a serovar not represented in the panel of reference strains.

It was found that 4 samples of the 13 kidney fragments homogenized in saline solution (31%) showed positive reactions in the PCR test, indicating the presence of *Leptospira* DNA fragments in those samples (Figure 1). The four positive samples were taken from the opossums identified as D-13, D-18, D-29 and

Table 1: Serology of Didelphis aurita opossums with positive reactions by the micro agglutination test (MAT).

Serovar	Serogroup	Strain	D-13	D-18	D-29	D-30
Australis	Australis	Ballico	Neg	Neg	Neg	Neg
Autumnalis	Autumnalis	Akiyami A	Neg	Neg	Neg	Neg
Castellonis	Ballum	Castellón 3	Neg	Neg	Neg	Neg
Bataviae	Bataviae	Swart	Neg	Neg	Neg	Neg
Canicola	Canicola	Hond Utrecht IV	Neg	Neg	Neg	Neg
Cynopteri	Cynopteri	3522 C	Neg	Neg	Neg	Neg
Grippotyphosa	Grippotyphosa	Moskva V	Neg	Neg	Neg	Neg
Hebdomadis	Hebdomadis	Hebdomadis	Neg	Neg	Neg	Neg
Icterohaemorrhagiae	Icterohaemorrhagagiae	RGA	Neg	Neg	Neg	Neg
Copenhageni	Icterohaemorrhagiae	M20	Neg	Neg	Neg	Neg
Javanica	Javanica	VeldratBatavia 46	Neg	Neg	Neg	Neg
Panama	Panama	Cz214	1:200	1:800	Neg	1:400
Pomona	Pomona	Pomona	Neg	Neg	Neg	Neg
Pyrogenes	Pyrogenes	Salinem	Neg	Neg	Neg	Neg
Hardjo	Sejroe	Hardjopratino	Neg	Neg	Neg	Neg
Sejroe	Sejroe	M84	Neg	Neg	Neg	Neg
Wolffii	Sejroe	3705	Neg	Neg	Neg	Neg
Tarassovi	Tarassovi	Perepeletsin	Neg	Neg	Neg	Neg
Patoc	Semaranga	Patoc 1	Neg	Neg	Neg	Neg
???	???	CLEP 00260	Neg	Neg	1:400	Neg
???	???	CLEP 00261	1:400	1:1,600	Neg	1:800

D-30. The higher sensitivity of the PCR test compared to serology and culturing would be expected due to the characteristics of the tests regarding to sensitivity and specificity. It should be stressed that leptospires colonize the renal tubules of infected animals being excreted intermittently through the urine of the carriers for long periods.

Two strains were isolated from the opossums identified as D-13 and D-29. The isolation of two strains out of 13 kidney samples (15,4%) would should be compared to the findings in similar exploratory studies focusing in wild animals [1]. It should be highlighted considering the indisputable evidence of the presence of *Leptospira* in opossums and the presumable circulation of *Leptospira* spp in that area. The isolated strains now identified as CLEP 00260 and CLEP 00261 are safeguarded and available at the *Leptospira* culture collection culture, WHO Collaborating Center for Leptospirosis, Oswaldo Cruz Institute/ FIOCRUZ (http://clep.fiocruz.br/index?services).

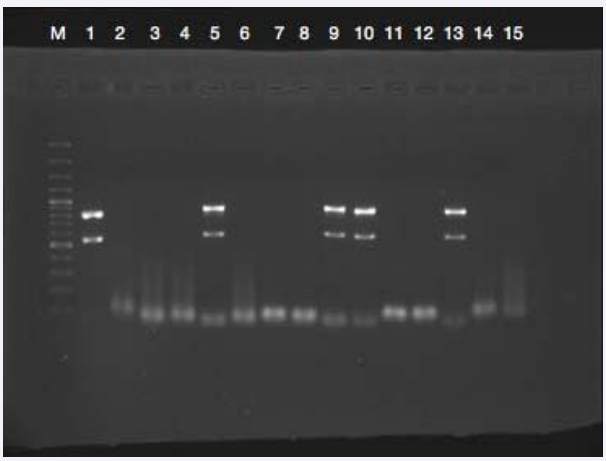

Figure 1 Agarose electrophoresis gel (1.5%) of multiplex-PCR showing the amplification of *lip*L41 (520bp) and *fla*B (793 bp) genes from kidney fragments of opossums *Didelhpis aurita*. M – molecular weight standard (O´Gene Ruler 100bp Plus; Thermo Scientific); lane 1 - positive control (*L. interrogans* serovar Copenhageni strain M20 DNA); lane 2 - negative control; lane 3 - opossumD-01 ; lane 4 D-04 ; lane 5- D-05; lane 6 D-10; lane 7 D-9 ; lane 8– D-16 ; lane 9 –D-13; lane 10 –D-18; lane 11 – D-15; lane 12 –D-20; lane 13 –D-29; lane 14 D-31 ; lane 15 - 35.

The preliminary serogrouping characterization by MAT demonstrated their possible antigenic relations to Panama and Grippotyphosa serogroups, respectively (data not shown). These data would be interpreted as indicative for further complementary tests.

The MLST analysis of both isolates could not reveal a 100% match with other sequence types deposited at the database. The closest match of strain CLEP 00260 was with ST 177, represented by a strain of *L.santarosai*. Concerning the strain CLEP 00261, the closest match was with STs 166 and 171, both represented by strains of *L. noguchii*. More refined molecular methods, such as secY sequencing and pulsed field gel electrophoresis should be used to improve the identification isolates and their taxonomic position at the levels of serovar and species.

It should be pointed out that to date the identification of *Leptospira* at the serovar or species level is not simple. The identification and taxonomic position are usually performed at international reference or research laboratories following international recommendations from experts such those published as a Position Stament-Speciation of Leptospiral Isolates shown at the International Leptospirosis Society (https://drive.google.com/file/d/1gYCph8eRuV3uUEnGi65_sx3MUpll8ShV/

view) and reports of the Subcommitee on the taxonomy of Leptospiracea from the International Committee on Systematics of Procaryotes [9].

No data were found about the isolation of *Leptospira* spp in *Didelphis aurita*, known as black ear possums. Prevalence data on other species considering serological data by MAT and isolation of Leptospira show substantial quantitative variations. The study metrics would not be comparable to quantitative assessments especially considering differences in sampling, time and geographical area [1,2]. They are well tolerant to anthropic interventions, sheltering in-house linings, hollow trees, between roots or under dry leaves, using the human food remains as a source of protein [6].

The importance of the described findings corroborates one of the most relevant hypotheses regarding the possibilities of transmission of *Leptospira* associated to wild focus of infection. The main concern here is the aspects related to transition areas in the limits of forest areas, where there are interactions between the animal carriers and areas of forests degradation resulting from human occupation. Other factors, such as geographic aspects that include lowland areas, nearby water sources (waterfalls, rivers and lakes) and climatic conditions that favor the occurrence of frequent floods, that are very common in tropical regions should be observed. The aspects mentioned are present in the area where the animals were captured. This context would be a model where the human-animal-ecosystem interface can be considered for possible prevention and control measures.

CONCLUSION

Didelphis aurita is a potential carrier of *Leptospira* spp. (presumptively *L. noguchi* and *L. santarosai*) in the Atlantic Forest, Rio de Janeiro, Brazil.

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