Original Article

Braz. J. Vet. Parasitol., Jaboticabal, v. 26, n. 1, p. 17-27, jan.-mar. 2017
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612017001

Leishmania in synanthropic rodents (*Rattus rattus*): new evidence for the urbanization of *Leishmania* (*Leishmania*) *amazonensis*

Leishmania em roedores sinantrópicos (*Rattus rattus*): uma nova evidência da urbanização de *Leishmania* (*Leishmania) amazonensis*

Eloiza Teles Caldart[1,2]*; Roberta Lemos Freire[1]; Fernanda Pinto Ferreira[1]; Bruno Bergamo Ruffolo[1]; Mônica Raquel Sbeghen[3]; Marcelle Mareze[1]; João Luis Garcia[2]; Regina Mitsuka-Breganó[2]; Italmar Teodorico Navarro[1]

[1] Laboratório de Zoonoses e Saúde Pública, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina – UEL, Londrina, PR, Brasil

[2] Laboratório de Parasitologia, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina – UEL, Londrina, PR, Brasil

[3] Laboratório de Imunologia Animal, Departamento de Ciências Patológicas, Universidade Estadual de Londrina – UEL, Londrina, PR, Brasil

Received August 30, 2016
Accepted October 25, 2016

Abstract

This study aimed to detect parasites from *Leishmania* genus, to determine the prevalence of anti-*Leishmania* spp. antibodies, to identify circulating species of the parasite, and to determine epidemiological variables associated with infection in rats caught in urban area of Londrina, Paraná, Brazil. Animal capture was carried out from May to December 2006, serological and molecular methods were performed. DNA was extracted from total blood, and nested-PCR, targeting SSu rRNA from *Leishmania* genus, was performed in triplicate. The positive samples were sequenced twice by Sanger method to species determination. In total, 181 rodents were captured, all were identified as *Rattus rattus* and none showed clinical alterations. Forty-one of the 176 (23.3%) animals were positive for *Leishmania* by ELISA and 6/181 (3.3%) were positive by IFAT. Nine of 127 tested animals (7.1%) were positive by PCR; seven were identified as *L.* (*L.*) *amazonensis*, one as *L.* (*L.*) *infantum*. Four rats were positive using more than one test. This was the first description of synanthropic rodents naturally infected by *L.* (*L.*) *amazonensis* (in the world) and by *L.* (*L.*) *infantum* (in South Brazil). Regarding *L.* (*L.*) *amazonensis*, this finding provides new evidence of the urbanization of this etiological agent.

Keywords: Leishmaniasis, Londrina, PCR, zoonosis, *Leishmania* (*L.*) *infantum*, *Leishmania* (*L.*) *amazonensis.*

Resumo

Esse estudo objetivou detectar parasitos do gênero *Leishmania*, determinar a prevalência de anticorpos anti-*Leishmania* spp., identificar as espécies circulantes do parasito e determinar variáveis epidemiológicas associadas com a infecção em ratos capturados em área urbana de Londrina, Paraná, Brazil. A captura dos animais ocorreu de maio a dezembro de 2006, métodos sorológicos e moleculares foram realizados. O DNA foi extraído do sangue total, uma *nested*-PCR cujo alvo foi o gene SSu rRNA do gênero *Leishmania*, foi realizado em triplicata. As amostras positivas foram sequenciadas duas vezes pelo método de Sanger para a determinação da espécie. No total, 181 roedores foram capturados, todos foram identificados como *Rattus rattus* e nenhum apresentou alterações clínicas. Quarenta e um dos 176 (23,3%) animais foram positivos no ELISA para *Leishmania* e 6/181 (3,3%) foram positivos na RIFI. Nove dos 127 animais testados (7,1%) foram positivos na PCR; sete foram identificadas como *L.* (*L.*) *amazonensis*, um como *L.* (*L.*) *infantum*. Quatro ratos foram positivos em mais de um teste. Essa é a primeira descrição de roedores sinantrópicos naturalmente infectados por *L.* (*L.*) *amazonensis* (no mundo) e por *L.* (*L.*) *infantum* (no Sul do Brasil). Com relação a *L.* (*L.*) *amazonensis*, esse resultado é uma nova evidência da urbanização desse agente etiológico.

Palavras-chave: Leishmanioses, Londrina, PCR, zoonoses, *Leishmania* (*L.*) *infantum*, *Leishmania* (*L.*) *amazonensis.*

***Corresponding author:** Eloiza Teles Caldart. Laboratório de Zoonoses e Saúde Pública, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina – UEL, Rodovia Celso Garcia Cid, 445, Km 380, CEP 86057-970, Londrina, PR, Brasil. e-mail: eloiza.vet@gmail.com

Introduction

The *Leishmania* parasite is a member of the Kinetoplastida class and Trypanosomatidae family, which comprises species that infect multiple mammalian species (GRAMICCIA, 2011; ROQUE & JANSEN, 2014). In nature, all species of this genus are transmitted to the vertebrate host by the bite of hematophagous females of several species of phlebotomine sand flies (class: Insecta, order: Diptera) (FORATTINI, 1973). These protozoan species have complex transmission cycles with region-specific epidemiological characteristics (ASHFORD, 1996; ROTUREAU, 2006). Infections by the aforementioned parasites can lead to diseases with a wide spectrum of clinical forms; these are collectively known as leishmaniosis, which are zoonosis and neglected infectious diseases (DUJARDIN et al., 2008; ALVAR et al., 2012).

Approximately 58,000 cases of visceral leishmaniosis (VL) are reported worldwide each year and six countries together account for more than 90% of the global estimated VL incidence: India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil (ALVAR et al., 2012). Between 2007 and 2015, 33,488 VL human cases were notified in Brazil and the mortality rate was 6.36% (SINAN, 2016). VL is caused by *Leishmania (Leishmania) infantum* (also known as *L. (L.) chagasi*), and its vectors are *Lutzomyia longipalpis* and *Lutzomyia cruzi*, the second being of limited importance (BRASIL, 2014). In the 1990s, approximately 90% of reported Brazilian VL cases occurred in the northeast region. However, this trend appears to be changing, as the disease reaches urban and peri-urban areas of all regions of the country (BRASIL, 2014). VL was not considered endemic to Paraná State until July 15, 2015, when the first autochthonous human case was reported (ANP, 2015).

Regarding cutaneous leishmaniosis (CL), 220,000 cases are reported each year worldwide and ten countries together account for 70 to 75% of its global estimated incidence; Brazil is also included in this list (ALVAR et al., 2012). The American cutaneous leishmaniosis (ACL) in Brazil represents a serious public health problem; it has been diagnosed in all states since 2003 (BRASIL, 2013). Between 2007 and 2015, 0.45% of human ACL cases in Brazil have occurred in the southern region, with Paraná State accounting for 57.2 to 98.0% (PONTELLO et al., 2013; SINAN, 2016). Autochthonous cases in dogs have been reported in Paraná state (CASTRO et al., 2007). The primary reservoirs of ACL disease-causing agents are small mammals, particularly wild rodents (BRANDÃO-FILHO et al., 2003; DANTAS-TORRES et al., 2010; ROQUE & JANSEN, 2014). Although ACL maintains a rural disease profile, its occurrence can also be found in urban areas; this is especially true with remaining forest or vegetation corridors associated with the local hydrography, and with primary and secondary riparian forests (NEGRÃO & FERREIRA, 2009; MEMBRIVE et al., 2012; CONSTANTINO et al., 2014).

Rattus rattus also known as roof rat is the predominant rodent in mostly rural areas of Brazil and it is also common in urban centers. These animals tend to live on high surfaces, and travel down to ground level in search of food and water. They live in groups, the size of which depends on existing resources (FUNASA, 2002; BONVICINO et al., 2008). The black rat is an exotic species introduced to the Americas during the colonization period, and has been suspected to serve as a reservoir for *Leishmania (Viannia) braziliensis* complex in Brazil (ROQUE & JANSEN, 2014); these animals are also potential sources of infection to peri domestic sandflies (BRANDÃO-FILHO et al., 2003). The production of huge quantities of waste, the reduction of areas available for disposal of these materials, and urbanization processes are examples of human actions that occur in many cities in Brazil and other countries. These activities create environments conducive to the spread of leishmaniosis as they increase the range and density of vectors and reservoirs.

The magnitude of health problems caused by leishmaniosis, combined with the complexity of its epidemiology, indicate a need to understand all stages in its transmission and develop effective control strategies (ROQUE & JANSEN, 2014). Solid waste recycling centers, investigated herein, offer environmental complexity and all relevant aspects for the study of *Leishmania*; this study aimed to investigate infections by *Leishmania* spp., identify circulating species of the parasite, and to determine epidemiological variables associated with infection in urban rats.

Methods

Location of study

The city of Londrina (23°18'36"S/51°09'46"W) is located in the north-central mesoregion of Paraná State, in the southern region of Brazil. It is 610 m above sea level and has a subtropical climate, with year-round rainfall concentrated mostly in the summer months. It has an average annual temperature above 21 °C and an estimated human population of 543,003 (IBGE, 2015).

Sampling

The points for collection of data and animals were the recycling centers of solid waste and junkyards from the five regions of the city; a starting point was taken as the surroundings of Basic Health Units (BHU). The sample size calculation was done using EpiInfo 3.5.3 (DEAN et al., 1990), for an infinite population; a prevalence of 50% was estimated, with a precision of 7.5% and a significance level of 5%, resulting in 171 samples. The work was designed to search for more than one parasite species; how we couldn´t choose just one prevalence value, we have chosen 50%, because it gives us the larger possible number of samples taking into account the other given fixed parameters (precision, confidence level and population size).

Animal trapping and sample collection

The rats were captured between the months May and December 2006, using cage mousetraps where the trigger is activated by bait placed in its interior. Traps were assembled at the end of the afternoon in areas where we found traces of rodents such as feces, fat stains, trails, or areas of plentiful food; we checked the traps the following morning. Rats were captured, identified by Prof. DSc Roberta Lemos Freire, and anesthetized for the collection of blood

and serum from the brachial plexus. After which, animals were euthanized. Half of the material was kept at room temperature until the clot reaction occurred (to obtain the serum), whereas the other half was taken for whole blood and frozen at -20 °C until use.

ELISA

The number of samples analyzed by each method varied according to the availability of the collected material. Serum samples were analyzed, in duplicate, by ELISA (enzyme linked immunosorbent assay) to determine the prevalence of anti-*Leishmania* spp. IgG antibodies as recommended by the Ministério da Saúde (MS) for screening the canine population. Antigen preparation and ELISA were performed according to Szargiki (2005) with minor modifications. In each well of the ELISA plates (Microlon 600, Greiner), 100 µL of *L. (L.) amazonensis* (MCAN/BR/2011/cãoLV01) total promastigote lysate (2.5 µg/mL), previously diluted in sodium carbonate–bicarbonate buffer (0.05 M, pH 9.6), was added. After overnight incubation at 4 °C, plates were washed three times with 0.9% saline containing 0.05% Tween-20, and were blocked for 1 h at 37 °C using 200 µL of 2% casein in phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween 20). After another wash, 100 µL of sera diluted (1:50) in PBS-Tween 20 was added to each well and incubated for 1 h at 37 °C. After another wash, 100 µL of anti-Rat IgG labeled with peroxidase (Sigma Aldrich - A9037) was added to each well and incubated for 1 h at 37 °C. The enzyme reaction was carried out with 100 µL of o-Phenylenediamine dihydrochloride (Sigma Aldrich - P8287) solution according to the manufacturer's instructions. The reaction was stopped by adding 50 µL of 1M H_2SO_4, and the optical density (OD) was read at 450 nm using an ELISA reader (iMark, Bio-Rad). Six negative and four positive controls were included in each plate, in duplicate, and values were expressed as the mean OD of the serum. The mean OD value from negative controls plus three standard deviations was considered as the cut-off point. The negative control was obtained from a young laboratory black rat, and the positive controls were from the research group of Prof. DSc. Mario Augusto Ono.

IFAT

Serum samples were analyzed by IFAT (immunofluorescence antibody test) according to Oliveira et al. (2008) to determine the prevalence of anti-*Leishmania* spp. IgG antibodies as recommended by the Ministério da Saúde (MS) for confirmation in canine populations. IFAT slides were prepared using *Leishmania* (*Leishmania*) *amazonensis* (MCAN/BR/2011/cãoLV01) promastigotes (OLIVEIRA et al., 2008). The concentration of anti-Rat IgG FITC (Sigma Aldrich - F6258) conjugates was standardized for the slides. Positive and negative controls were included in all tests; the negative control was obtained from a young black laboratory rat, and the positive control was from the research group of Prof. DSc. Mario Augusto Ono. In positive serum samples, parasites displayed a bright-green peripheral stain and the considered cutoff point was a titer of 20 (BEN-ISMAIL et al., 1989; CÁSSIA-PIRES et al., 2014). Positive samples were serially diluted two-fold to obtain a bigger reagent titer.

DNA extraction

The DNA extraction was performed from whole blood of synanthropic rodents using the commercial QIAmp DNA Blood Mini Kit (Qiagen), according to the manufacturer's recommendations. Negative controls for the DNA extraction process were used in all assays, included for every ten samples, and were tested with the samples to identify possible contamination of samples during the DNA extraction procedure. The extracted DNA was eluted with 50 µL of elution buffer in a sterile 1.5mL tube, identified for use in PCR reactions, and stored at -20 °C. The DNA extraction yield was determined by measuring the absorbance at 260 nm in a Picodrop (Thermo Fisher Scientific, Wilmington, DE, USA) spectrophotometer and the A260/A280 absorbance ratio was assessed to verify the purity of the obtained DNA.

Molecular detection

To detect *Leishmania* parasites, we utilized a nested-PCR (nPCR) assay targeting the small subunit of ribosomal RNA (SSu RNAr) gene fragment, which is within a region that is highly conserved among *Leishmania* species. The first amplification step was performed with 2 µL of DNA solution added to 23 µL of PCR mix containing 0.2 mM deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Sweden), 2 mM $MgCl_2$, 5 mM KCl, 75 mM Tris-HCl, pH 9, 1 U Platinum Taq DNA Polymerase (Life Technologies) and 20 pmol of specific primers (R221 and R332) (VAN EYS et al., 1992). The amplification conditions were denaturation at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 60 °C, and 72 °C, and a final extension at 72 °C for 5 min. Two microliters of the resulting amplification product was used as a template in the second reaction in the presence of *Leishmania* genus specific primers (R223 and R333) (VAN EYS et al., 1992). The reaction conditions were the same as before except for an increased annealing temperature of 65 °C. The final reaction resulted in a 353bp fragment that was visualized after electrophoresis on 1.5% agarose gel (Invitrogen) stained with SyBr safe DNA stain (Invitrogen). Every set of reactions included a PCR-negative control (ultrapure water), an nPCR-negative control (ultrapure water), and a positive control (*L.* (*L.*) *infantum* MHOM/BR/75/M2903DNA or *L.* (*L.*) *amazonensis* MCAN/BR/2011/cãoLV01). All samples were tested in triplicate to improve sensitivity, as we were using DNA extracted from total blood as a template.

Parasite species characterization

The SSu rRNA PCR products were purified using a QIAquick PCR Purification kit (QIAGEN), and quantified using a Picodrop (Thermo Fisher Scientific, Wilmington, DE, USA). Direct Sanger sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, USA) with the corresponding forward and reverse primers, in the 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, USA), according to the

manufacturer's instructions. The obtained sequences were examined with PHRED software (EMBRAPA, 2016) for quality analysis of chromatogram readings. The sequences were accepted if base quality was equal to or higher than 20. Consensus sequences were determined by CAP3 software (EMBRAPA, 2016) and the sequence identity was verified by comparison to sequences deposited in GenBank using BLAST software (NCBI, 2016).

To distinguish *L.* (*L.*) *amazonensis* from *L.* (*L.*) *mexicana* we performed another PCR using extracted DNA from positive rats in SSU rRNA PCR. Polymorphism-specific primers (a1: 5′-TGCGAGGATAAAGGGAAAGAA-3′ and a2: 5′-GTGCCCTGACTTGCATGTCTA-3′) targeting the p53 gene were used according to Mimori et al. (1998). These primers amplify *L.* (*L.*) *amazonensis* and do not amplify *L.* (*L.*) *mexicana* because of a difference between these two species in the 3′ region of primer a1. If *L. (L.) amazonensis* is present, the PCR will result in a 62bp fragment, if not the PCR is going to be negative.

Research tools

To obtain epidemiological data concerning parameters such as environment, sanity, species, age, and gender a questionnaire was used; the association of these factors was later made with the obtained results.

Statistical analysis

Data tabulation was performed using EpiInfo 3.5.3 (CDC, Atlanta). Statistical significance between questionnaire variables and leishmanosis were evaluated by chi-square (x2) or Fisher's exact tests with a 5.0% significance level.

Nucleotide sequence data

Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers: KX011478, KX011479, KX011480, KX011481, KX011482, KX011483, KX011484, KX011485.

Ethical considerations

This study was approved by the Comitê de Ética em Experimentação Animal (CEEA) of the Universidade Estadual de Londrina (nº 28/2006).

Results

A total of 181 rodents were captured, and all of them were identified as *Rattus rattus* species; 104/181 (57.5%) were female and 150/181 (82.9%) were adults. The animals were apparently healthy and showed no visible ectoparasites upon visual inspection. These animals were captured at 35/35 collection sites (100%), distributed in north (7), south (6), east (6), west (7), and central (9) regions of Londrina city. The region with the highest number of captured rats was the central with 62/181 catches (34.3%). Owners or workers of all sites visited reported the presence of rats at night and at any establishment we found workers using of personal protective equipment (PPE). Dogs lived at 29/35 capture sites (82.8%), cats at 2/35 (5.7%), and chickens at 2/35 (5.7%).

Regarding serological testing of rats, 41/176 (23.3%) were positive by ELISA, but only two samples showed expressive OD; the others were very close to the cutoff point. Of the 41 positives, 28 (68.3%) were female and 39 (95.1%) were adults. Data analysis revealed an association between *Leishmania* positivity (by ELISA) and age (p=0.027); a higher proportion of adult animals were positive than young animals. Using IFAT, 6/181 (3.3%) rats were positive; two had a titer equal to 20, three had a titer equal to 40 and one had a titer equal to 80. All positive were female and adult. Data analysis revealed an association between IFAT positivity in rats and the free access of other animal species in the places of rodent collection (p=0.032). From 127 rats analyzed by molecular methods, nine (7.1%) were positive by SSU rRNA PCR. Three of nine positive rats were captured at the same address (Figure 1). Of the nine positive, six (66.7%) were female and six (66.7%) were adults. PCR data analysis revealed no association between leishmaniosis in rats and all other variables tested. Considering all diagnosis methods used in this study, we had 51 positive animals, three rats were positive in two tests, one was positive using all methods. In this population, the positive proportion of female rats was higher than that of male rats when considering all three diagnostic methods (p=0.038). The 353 bp fragments from SSu rRNA PCR, from the nine positive animals, were purified and sequenced twice by the Sanger method; seven samples were identified as *L.* (*L.*) *amazonensis* or *L.* (*L.*) *mexicana*, and one was identified as *L.* (*L.*) *infantum* (Figure 2); one could not be sequenced. We further confirmed *L.* (*L.*) *amazonensis* detected samples by performing a1/a2 polymorphism-specific PCR (PS-PCR) described by Mimori et al. (1998).

Discussion

The high prevalence of rodents, captured in solid waste recycling sites, indicates the existence of shelter, food and water, essential components for habitation and proliferation of rodents. The dispersion of the species *R. rattus* is 50 m (FUNASA, 2002), which is indeed worrisome as 15 of the 35 sites selected for this study contained residences. Any animal caught in this study presented no signs of disease, based on clinical examination, which was also reported in previous studies (OLIVEIRA et al., 2005; AKHAVAN et al., 2010; MARCELINO et al., 2011; LIMA et al., 2013).

Regarding serological methods, we determined 41/176 (23.3%) of animals to be positive by ELISA and 6/181 (3.3%) by IFAT. The literature is scarce regarding serological diagnosis of leishmaniosis in rats; we could only find two studies using an immunochromatographic method (LIMA et al., 2013; SINGH et al., 2013), two using ELISA (MUKHTAR et al., 2000; BARBOSA, 2005) in Sudan and Brazil, and two using IFAT (BEN-ISMAIL et al., 1989; CÁSSIA-PIRES et al., 2014) in Tunisia and Brazil. Our prevalence based on ELISA was higher (23.3%) than previously reported (0% in Brazil and 4.1% in Sudan) and based

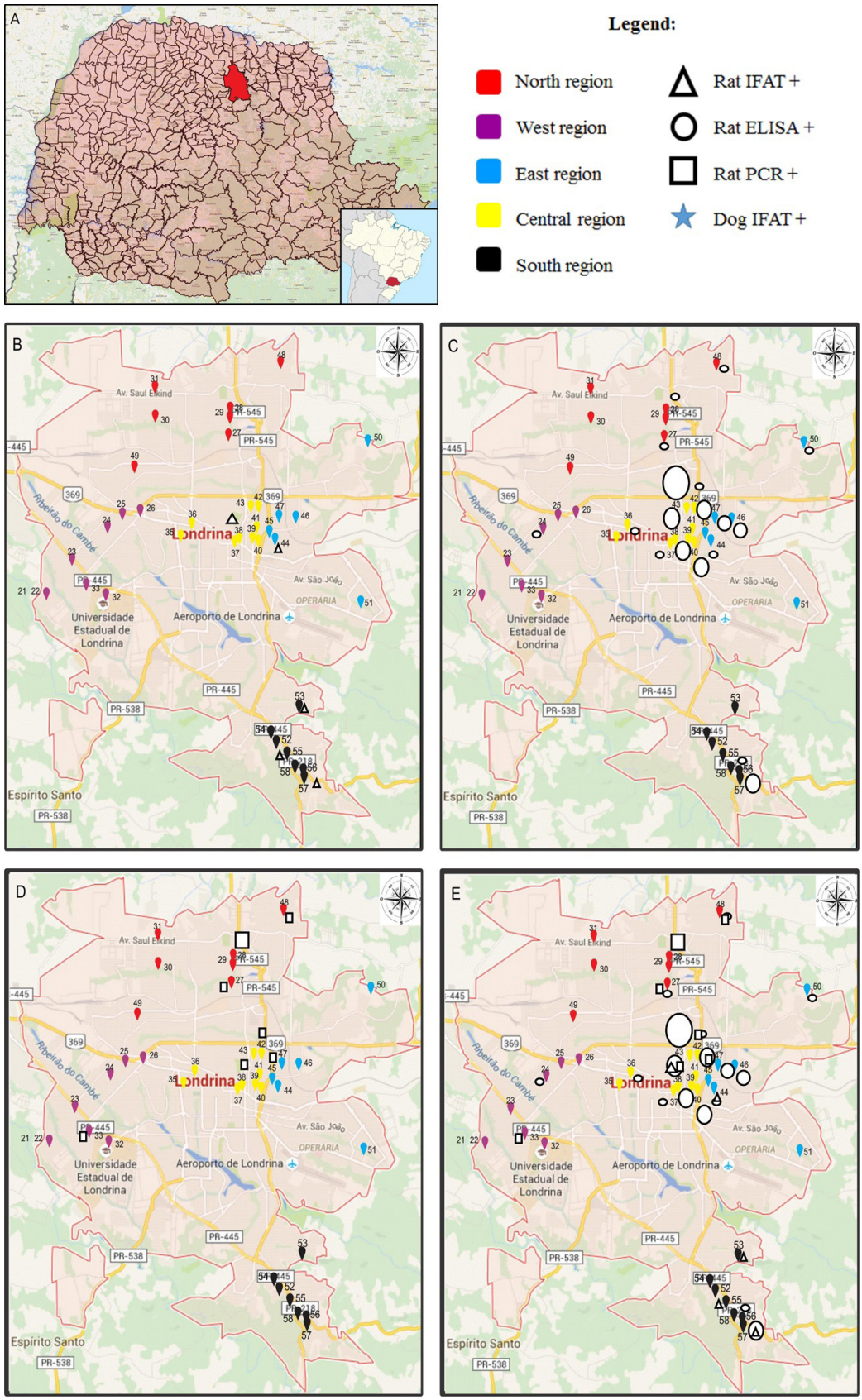

Figure 1. (A) Geographic localization of Londrina city in Paraná State and in Brazil; (B) Geographic distribution of collection sites and positive rats determined by IFAT; (C) Geographic distribution of collection sites and positive rats determined by ELISA; (D) Geographic distribution of collection sites and positive rats determined by PCR; (E) Geographic distribution of collection sites and positive rats determined by IFAT, ELISA, and PCR. The sizes of the circles, triangles, and rectangles represent the number of positive rats at each collection point.

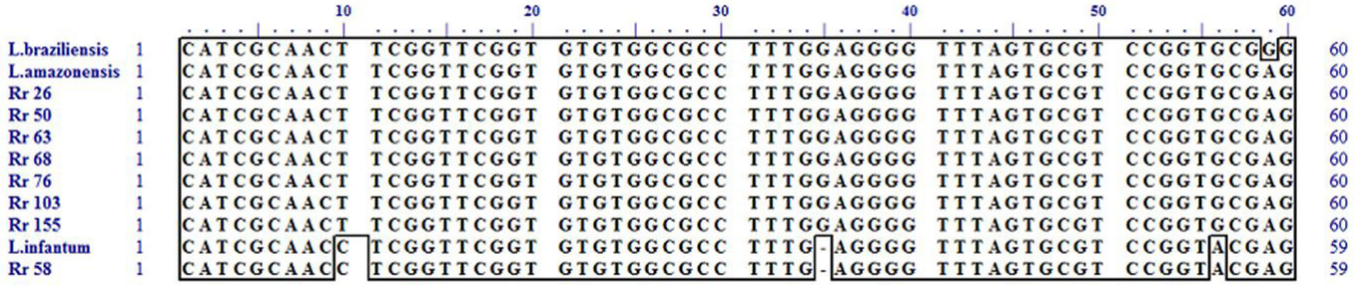

Figure 2. Partial alignment of nucleotides from SSu RNAr fragment of *Leishmania* spp. DNA from positive samples of *R. rattus* (Rr26, Rr50, Rr58, Rr63, Rr68, Rr76, Rr103, and Rr155) compared to *L. (L.) amazonensis* (M80293), *L. (L.) infantum* (M81430) and *L. (V.) braziliensis* (M80292) reference strains. Polymorphisms in positions 10, 35, and 56 differentiate *L. (L.) infantum* from *L. (L.) amazonensis* and *L. (V.) braziliensis*. Polymorphism in position 59 differentiates *L. (L.) amazonensis* from *L. (V.) braziliensis*.

on IFAT was lower (3.3%) than that of previous reports (27.2% in Tunisia and 51.3% in Brazil). The PCR prevalence was nine of 127 animals (7.1%), which is a low prevalence upon comparing to other studies performed in Brazil (BRANDÃO-FILHO et al., 2003; OLIVEIRA et al., 2005; QUARESMA et al., 2011; LIMA et al., 2013; CARDOSO et al., 2015; FERREIRA et al., 2015), South America (ALEXANDER et al., 1998; LIMA et al., 2002), Iran (AKHAVAN et al., 2010; DAVAMI et al., 2013; MIRZAEI et al, 2014), Saudi Arabia (IBRAHIM et al., 1992), Italy (ZANET et al., 2014), Spain (NAVEA-PÉREZ et al., 2015), and Portugal (HELHAZAR et al., 2013). This low prevalence in PCR could be explained by our use of total blood as sample; most other studies used both blood and tissue (liver, spleen, skin, bone marrow). In few cases, the positivity rate of blood samples was higher than that of tissues (OLIVEIRA et al., 2005; MARCELINO et al., 2011). Neitzke-Abreu et al. (2013) tested the accuracy of some PCR-based techniques for CL diagnosis in humans and concluded that the use of blood (buffy coat) may be indicated for patients with no lesions. Our prevalence was higher than reported in other Brazilian (CÁSSIA-PIRES et al., 2014; LARA-SILVA et al., 2014) and foreign studies (LIMA et al., 2002; PAPADOGIANNAKIS et al., 2010; MENDONÇA et al., 2011; SHENDER et al., 2014; VLADIMIR et al., 2015). One study analyzed rodents from waste sites (VLADIMIR et al., 2015) in Croatia; 1/173 (0.5%) rodent (*R. rattus*) tested positive for *L.* (*L.*) *infantum*; the authors identified five species of phlebotomine sand flies at those places.

Cássia-Pires et al. (2014) demonstrated infection rates of 51.3%, using IFAT, and suggest that natural *Leishmania* infection is much higher than that observed using molecular diagnosis (4.6%). In contrast, Lima et al. (2013), through serological testing, revealed an overall positivity of 5.0%, whereas PCR yielded 28.6% positivity, and all seropositive animals were PCR negative. Regarding this lack of concordance between diagnostic methods, serological reacting samples thought to be from animals previously exposed to *Leishmania* parasites and are expected to be still infected. However, parasites have non-uniform distribution in tissues and blood of vertebrate hosts, and may not be present in analyzed tissues, resulting in a false negative through PCR. Furthermore, positive animals based on molecular diagnostics, but negative based on serology, may have been caught in an initial phase of infection before the production of detectable IgG, required for serological tests. Alternatively, it is crucial to take into account that the presence of other trypanosomatids was previously shown to lead to cross-reactions (DÍAZ-SÁEZ et al., 2014).

In the present work, five of nine positive animals by PCR were from the north region of Londrina city, three of them from the same address (collection point 28). At collection point 48 (north region), Costa et al. (2016) discovered a dog positive for leishmaniosis by IFAT and we discovered a PCR positive rat (Figure 1). This was a region with higher geographic expansion with a large number of illegally occupied areas in 2006 (IPPUL, 2008), the year of sample collection. This factor may explain why those animals had recent infections. The overall positivity, including that based on serological methods, was concentrated in the east (42.3%) and central (35.4%) regions. Although these collection points were in different regions, they were very close (Figure 1). At collection point 38 (central region) we had two rats test positive only by ELISA, one was positive by IFAT and ELISA, one was positive by ELISA, IFAT, and PCR, and another dog was positive by IFAT (COSTA et al., 2016); this demonstrates a trend that *Leishmania* detection had hot spots in Londrina city (Figure 1).

This study demonstrated statistically more adult animals positive through ELISA than young animals in the rodent population. These findings are likely explained by increased exposure to infected vectors throughout the rats' lives. Data analysis revealed a statistical association between IFAT positivity in rats and places where other animal species were not allowed to enter, for example, dogs, cats and, chickens (TANURE et al., 2015). In other words, the presence of animal species different from rats was a protection factor against leishmaniosis in rats (OR= 0.01273 to 0.8341). A study on *Nyssomyia neivai* species broadly found in Paraná State (MEMBRIVE et al., 2004; DIAS-SVERSUTTI et al., 2007; SILVA et al., 2008; CERINO et al., 2009; CRUZ et al., 2013; MELO et al., 2013), performed in Ribeira Valley São Paulo State, examined 988 engorged females, and no rat blood was detected in the phlebotomines (MARASSÁ et al., 2013). According to Dias-Sversutti et al. (2007), *N. neivai* and *N. whitmani* are opportunists and probably adjust their feeding habits based on the availability of hosts.

R. rattus can be found in the domicile (BRANDÃO-FILHO et al., 2003; QUARESMA et al., 2011), in the peridomicile

(BRANDÃO-FILHO et al., 2003; FERREIRA et al., 2015), in waste areas (VLADIMIR et al., 2015), in rural areas (ALEXANDER et al., 1998; BRANDÃO-FILHO et al., 2003; QUARESMA et al., 2011; FERREIRA et al., 2015), and in wild areas (RICHINI-PEREIRA et al., 2014; ZANET et al., 2014). Because of this adaptability, this rat species may be the link between sylvatic and peridomestic transmission cycles of leishmaniosis (BRANDÃO-FILHO et al., 2003; ANDRADE et al., 2015). We suggest that it may be the source of infection for peridomestic phlebotomine sand flies, specifically in cases of less anthropophilic vectors.

Despite difficulties, species-specific diagnosis is crucial to a better understanding of the complex network of transmission for *Leishmania* species (VAN DER AUWERA & DUJARDIN, 2015). *R. rattus* has previously been found to be naturally infected with *L. (L.) infantum*, *L. (L.) donovani*, *L. (L.) mexicana*, and *L. (V.) braziliensis* in several places around the world such as Brazil (ALENCAR et al., 1960; BRANDÃO-FILHO et al., 2003; SVOBODOVÁ et al., 2003; OLIVEIRA et al., 2005; QUARESMA et al., 2011; LIMA et al., 2013) and other countries (LIMA et al., 2002; ZULUETA et al., 1999; DAVAMI et al., 2013; EL ADHAMI, 1976; IBRAHIM et al., 1992; POZIO et al., 1981; DI BELLA et al., 2003; ZANET et al., 2014; NAVEA-PÉREZ et al., 2015; VLADIMIR et al., 2015 and SINGH et al., 2013). As in this work, other studies have found rats captured in the same place with more than one *Leishmania* species: Lima et al. (2002) found *L.* (*L.*) *mexicana* and *L.* (*V.*) *braziliensis*, and Ferreira et al. (2015) found *L.* (*L.*) *infantum* and *L.* (*V.*) *braziliensis*; which is very interesting from an epidemiological point of view.

We found one animal naturally infected with *L. (L.) infantum*; however, North Paraná has not reported any authochnous cases of visceral leishmaniosis in dogs or humans caused by this agent. There have also not been any reports of its vector. We have three hypotheses to address this issue (from most to least likely): (i) railway lines pass by Londrina north region carrying grains, which may have brought infected rats from São Paulo State; (ii) the vector is already endogenous to Londrina but new phlebotomine studies are needed to prove this; (iii) Londrina has a new vector that is transmitting *L. (L.) infantum*. In Paraná State, *L. longipalpis* was just discovered by Santos et al. (2012) in Foz do Iguaçu (500 km from Londrina). According to D'Andrea et al. (2015), canine leishmaniosis is spreading fast from the western counties of São Paulo State (Presidente Prudente, 167 km from Londrina) toward the border of Paraná State, and this is the most likely path to Londrina city to be followed by etiological agents and by the vector of visceral leishmaniosis.

This work is the first description of synanthropic rodents naturally infected by *L. (L.) amazonensis* in the world. This result corroborates an autochthonous case report of canine visceral leishmaniosis by *L. (L.) amazonensis* in Londrina published by our research group in 2012 (HOFFMANN et al., 2012) and an autochthonous case report of human cutaneous leishmaniosis by *L. (L.) amazonensis* in Maringá (80 km from Londrina) published in 1990 (SILVEIRA et al., 1990). Until the present work, this *Leishmania* species was only found naturally infecting wild rodents (LAINSON & SHAW, 1968; LAINSON & SHAW, 1970; CATARINO, 1998; TELLERIA et al., 1999). Clear expansion of leishmaniosis by *L. (L.) amazonensis* around the country has been observed over the last decades; according to Câmara-Coelho et al. (2011) 8% of American cutaneous leishmaniosis cases in Brazil are caused by the above species. Several recent scientific studies describe this expansion; in terms of hosts, Souza et al. (2005), Dorval et al., (2010) and Cardoso et al. (2015) reported *Felis catus domesticus* (domestic cat), *Mesocricetus auratus* (hamster) and *Necromys lasiurus* (wild rodent) infection. In terms of geographical distribution, Dorval et al. (2006), Marlow et al. (2013) and Carvalho et al. (2013) reported its detection in Mato Grosso do Sul, Santa Catarina and Rio de Janeiro states, Brazil. Regarding clinical manifestations, Barral et al. (1991) and Oliveira et al. (2007), reported *L. (L.) amazonensis* causing visceral leishmaniosis in humans in Bahia and Maranhão, and Tolezano et al. (2007), Dias et al. (2011) and Hoffmann et al. (2012) reported *L. (L.) amazonensis* causing visceral leishmaniosis in dogs in São Paulo, Minas Gerais, and Paraná States.

The known phlebotomine sand fly vector species of *L. (L.) amazonensis* are *L. flaviscutellata*, *L. reducta*, and *L. olmeca nociva* (FREITAS et al., 1989; LAINSON & SHAW, 1968; BRASIL, 2013). These vectors are less anthropophilic, which explains a lower frequency of human infection with this *Leishmania* species. Still corroborating with our hypothesis of *L. (L.) amazonensis* urbanization, *Lu. flaviscutellata* was found for the first time infected with it in a Brazilian urban area by Brilhante et al. (2015) in Bonito city, Mato Grosso do Sul state. A recent study (CARVALHO et al., 2014) using ecological niche modeling predicts southward expansion of *L. flaviscutellata* in South America because of climate change. Several studies have investigated phlebotomine fauna in Paraná State (GOMES & GALATI, 1977; TEODORO et al., 1993; TEODORO et al., 1999; OLIVEIRA et al., 2000; DIAS-SVERSUTTI et al., 2007; SILVA et al., 2008; CERINO et al., 2009; REIS et al., 2011; CRUZ et al., 2012; SANTOS et al., 2012; MEMBRIVE et al., 2012; CRUZ et al., 2013; MELO et al., 2013); none reported *L. (L.) amazonensis* vectors. Taking into account two autochthonous cases (one human and one canine) of leishmaniasis by *L. (L.) amazonensis* in North Paraná, more studies about phlebotomine fauna must be made in this region, now using Disney traps with rodents as baits (DORVAL et al., 2007), because *L. flaviscutellata* is strongly attracted to rodents (LAINSON & SHAW, 1968; SHAW & LAINSON, 1972; CARVALHO et al., 2014).

Conclusion

This is the first description of synanthropic rodents naturally infected by *L. (L.) amazonensis* in the world, and by *L. (L.) infantum* in South Brazil. Regarding *L. (L.) amazonensis,* this finding is new evidence of the urbanization of this etiological agent, emphasizing the spread of leishmaniosis in Brazil and its neglected disease situation.

Acknowledgements

We are grateful to CAPES, CNPq and Fundação Araucária for financial support, to Thaís Gomes Verzignassi da Silveira for providing the DNA for the positive controls and to Mario

Augusto Ono and Rafaela Macagnan for providing the serum for the positive controls.

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