



## Molecular detection of *Leishmania braziliensis* in *Rattus norvegicus* in an area endemic for cutaneous leishmaniasis in Brazil

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### ABSTRACT

*Leishmania* nested PCR (LnPCR) targeted to the *SSUrRNA* gene and DNA sequencing were used to analyze 315 tissue samples from 80 *Rattus norvegicus* specimens trapped in an area endemic for leishmaniasis in Belo Horizonte, Minas Gerais, Brazil. Of the samples analyzed, 17.46% (55/315) of all tissues, 10% (8/80) of skin, 26.92% (21/78) of blood, 30.76% (24/78) of bone marrow and 2.53% (2/79) of spleen were positive for *Leishmania*. The overall infection prevalence was 36.25% (29/80). The DNA sequencing showed that 65.51% (19/29) of the positive animals were infected by parasites belonging to the *Leishmania braziliensis* complex. The identification of *L. braziliensis* DNA in *R. norvegicus* in an area with a high prevalence of leishmaniasis might imply a zoonotic role of this species. The rodent control programs and health education may represent important measures toward the control of leishmaniasis.

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### 1. Introduction

In Brazil, leishmaniasis had been a disease that was restricted to rural areas but, after the 1980s, showed significant geographic expansion, with extensive adaptation of parasites (*Leishmania*) and vectors (Marzochi and Marzochi, 1994). The life cycle of the parasite involves several vertebrate hosts that can act as reservoirs, including rodents, edentates (armadillos, anteaters and sloths), marsupials (opossums), dogs and primates (Lainson and Shaw, 1987). American cutaneous leishmaniasis (ACL) has multiple etiological agents that have been isolated in Brazil, such as *L. braziliensis*, *Leishmania guyanensis*, *Leishmania amazonensis*, *Leishmania mexicana*, and *Leishmania lainsoni* (Lainson, 2010).

ACL occurs in all of the states of Brazil, and the number of human cases has grown steadily over the last twenty years, with an average of 26,402 cases per year (Sinan, 2010). From 2006 to 2009, Minas Gerais had 5338 cases of ACL, with 164 (3%) cases occurring in Belo Horizonte, a city with a very small rural area. In Belo Horizonte, *L. (Viannia) braziliensis* is the main species detected in cases of leishmaniasis (Passos et al., 1999). Transmission of peridomestic *L. braziliensis* depends on vector adaptations to human modifications to the environment. A survey conducted on sand flies by Souza et al. (2004) demonstrated that the peri- and intra-household *L. braziliensis* vectors, *Lutzomyia intermedia* and *Lutzomyia whitmani*, were present in all districts of Belo Horizonte.

Natural infection of synanthropic rodents, such as *Rattus rattus*, by *Leishmania* has been reported in the Old World (Hoogstraal et al., 1963; El-Adhami, 1976; Pozio et al., 1981; Ibrahim et al., 1992) and in the New World (Alencar et al., 1960; Brandão-Filho et al., 2003; Oliveira et al., 2005). However, there are few studies on *R. norvegicus*, and the results

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obtained thus far do not clarify the role of this species in the epidemiological chain of leishmaniasis (Giannini, 1985; Di Bella et al., 2003; Papadogiannakis et al., 2009; Motazedian et al., 2010; Psaroulaki et al., 2010).

Currently, molecular methods such as polymerase chain reaction (PCR) are widely used to detect *Leishmania* in clinical samples from humans or in domestic and synanthropic wild animals (Osman et al., 1997; Brandão-Filho et al., 2003; Oliveira et al., 2005; Silva et al., 2005; Wynsberghe et al., 2009), replacing laborious techniques, such as isolation in culture media which however still represents the only method to confirm host infection with viable parasites (Noyes et al., 1998; Evans et al., 1990; Akhavan et al., 2010).

The aim of this study was to detect *Leishmania* in *R. norvegicus* captured in the urban area of Belo Horizonte, in districts with a high prevalence of leishmaniasis.

## 2. Materials and methods

### 2.1. Study site

The study was conducted in Belo Horizonte, the capital of Minas Gerais, Brazil. Belo Horizonte was selected due to the high prevalence of cutaneous and visceral leishmaniasis in this city over the past several years. The study area was limited to the Pampulha, Venda Nova and North districts where 56 human cases of cutaneous leishmaniasis were recorded in the last four years (Sinan, 2010).

### 2.2. Capture of rodents

All procedures for handling animals were performed according to the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and were approved by the Ethics Committee on Animal Experiments (CETEA) (University protocol number 054/08). Animals were trapped in a galvanized wire cage (Tomahawk model, 35 cm × 12 cm × 12 cm), using dog food suspended in the cage as bait. Traps were distributed among ten locations, with a minimum distance of 200 m between them, and each trapping station was positioned at night and collected at dawn. Catches were carried out twice per week from July to November 2007 and April to November 2008. For the purpose of registering and classifying the animals, data on weight, length (tail and body) and the presence or absence of skin lesions were collected. Species identification was conducted (Bonvicino et al., 2008) by examining morphological characteristics according to specific guidelines.

### 2.3. Collection of samples and DNA extraction

Animals were sedated with 1–5 mg/kg of Xylazine and washed in a solution of 70% ethanol before collecting samples. Blood was collected by cardiac puncture, transferred to sterile tubes containing EDTA and stored at –20 °C until use. After blood collection, animals were euthanized by intraperitoneal injection of 50 mg/kg thiopental, and tissues (spleen, skin, tail and bone marrow) were harvested. Portions of each tissue were removed with the aid of single use forceps, scissors and scalpel blades placed in sterile

tubes containing 100% ethanol and stored at –20 °C until PCR was completed. To isolate DNA from blood and bone marrow, we used the Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare), according to the manufacturer's instructions. DNA from the spleen and skin were extracted using the GenomicPrep Cells and Tissue DNA Isolation Kit (GE Healthcare), following the protocol described by the manufacturer. Each DNA sample was eluted in 200 µl of warmed (70 °C) elution buffer and stored at –20 °C until use.

### 2.4. Detection of *Leishmania* DNA

To detect *Leishmania* infection, we utilized a nested PCR (LnPCR) assay targeting a SSUrRNA gene fragment, which is within a region that is highly conserved among *Leishmania* species. The LnPCR assay was followed by sequencing to identify the parasite species.

The primers used for the LnPCR assay were as follows: (R221): 5'GGT CCT TCC TTT GAT TTA CG-3'; (R332): 5'GGC CGG TAA AGG CCG AAT AG-3'; (R223): 5'TCC CAT GCC AAC CTC GGTT-3'; and (R333): 5'GGC GCG AAA GCG GTC CTG-3', according to the protocol developed by Van Eys et al. (1992) and adapted and modified by Cruz et al. (2002). Briefly, the first reaction was performed in a final volume of 50 µl containing 10 µl of DNA template and 40 µl of a PCR mix of 10X buffer with 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 15 pmol each of primers R221 and R332, and 1.4 units of Taq DNA polymerase (BioTools, Spain). Each set of reactions included three negative controls (containing all components except DNA) and one positive control (DNA purified from reference strains of *L. braziliensis* (MHOM/BR/1975/M2903)), *L. chagasi* (MHOM/BR/74/PP75) and *L. amazonensis* (MHOM/BR/LTB16). Cycling parameters were as follows: initial denaturation at 94 °C for 5 min, followed by 30 s at 94 °C, 60 °C and 72 °C for 30 cycles, with a final extension for 5 min at 72 °C. The first reaction generated a 603-bp band. The amplified products were diluted 1:4 in deionized water and used as the template for the next reaction. The final volume of the second reaction was 25 µl, containing 10X buffer with 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 15 pmol each of primers R223 and R333, 0.7 units of Taq DNA polymerase (BioTools, Spain) and 10 µl of template. Cycling parameters were the same as above with one exception: the annealing temperature was raised to 65 °C.

The final reaction produced a 353-bp fragment. PCRs were visualized by electrophoresis on a 1% agarose gel at 100 V in 0.5X TBE (0.045 M Tris-borate, 1 mM EDTA) and stained with ethidium bromide (0.5 mg/ml).

### 2.5. Endogenous control for PCR

Ten percent of the samples that were negative for each tissue were randomly selected and subjected to PCR, as described by Ferreira et al. (2010), to amplify the interphotoreceptor retinoid-binding protein (IRBP) gene, which is highly conserved among small mammals. This PCR assay tested the integrity of DNA extracted from the samples that were negative by LnPCR. The primers used in this reaction were as follows: fwd IRBP 5'TCC ACC ACC AAC TGC ACT GAG ATC CC 3' and rev IRBP 5'GTG GCT AGG AAG AAA

TGG GAC CC 3', which yielded a 227-bp fragment. The following cycling conditions were used: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, 72 °C for 1 min and then a final extension at 72 °C for 5 min. The amplicons were visualized on 1.5% agarose gels stained with ethidium bromide (0.5 mg/ml).

## 2.6. Sequencing of genomic DNA

To identify the species of *Leishmania* present in positive samples, sequencing of the 353-bp amplicons was performed, with 5 µl of the purified sample (template) added to a solution containing 1 µl of PREMIX (Big Dye® Terminator V3.1 Cycle), 3.2 pmol of primer (R223 or R333) and 3 µl of H<sub>2</sub>O. The cycling conditions were as follows: 96 °C for 1 min, 30 cycles of 96 °C for 15 s and 57 °C for 15 s (the optimal annealing temperature for the R223 and R333), with a final extension at 60 °C for 4 min. Sequencing was performed on an ABI 310 DNA Sequencer (Life Technologies). To distinguish between complexes of *Leishmania*, *L. braziliensis*, *L. infantum* (*L. chagasi*) and *L. amazonensis*, which all have been isolated in the study area editing, alignment and restriction mapping of the sequences were performed using the program BioEdit.

## 2.7. Statistical analysis

The difference between the frequencies of positive tissues and their relationship to gender and age was analyzed by the chi-square test (EpiInfo 3.5.3, Centers for Disease Control and Prevention, Atlanta), with a significance level of 5%.

## 3. Results

### 3.1. Collection of rodents

Eighty specimens of *Rattus norvegicus* were caught in the Pampulha, Venda Nova and North Sanitary districts; of these, 36.25% were adult females, 10% were juvenile females, 40% were adult males and 13.75% were juvenile males, according to the WHO classification scheme (1987). All animals were captured in peridomestic environments. Except for one animal that had a lesion at the base of the tail, all other animals showed no signs of infection or lesions.

### 3.2. Detection of *Leishmania*

A total of 315 samples from different tissues were analyzed, and 2.53% of spleen (2/79), 10% of tail skin (8/80), 26.92% of blood (21/78) and 30.76% of bone marrow (24/78) samples were positive by LnPCR. The infection rate of *R. norvegicus* was 36.25% (29/80). Of the 29 infected animals, 24.15% (7/29) had only one tissue test positive, 65.5% (19/29) had two, 6.9% (2/29) had three, and 3.45% (1/29) had all of the tissues analyzed test positive. A subset of the samples that were negative by LnPCR was also tested for the presence of the IRBP gene, and all were positive. The chi-square test revealed no significant correlation between

positivity by LnPCR and characteristics such as the gender and age of the animals.

Positive samples with sharp bands were selected, purified and sequenced. *Leishmania* species identification was possible in 65.51% (19/29) of the animals that tested positive by LnPCR, and all were identified as belonging to the *L. braziliensis* complex.

## 4. Discussion

Molecular methods (LnPCR and sequencing) were used to detect *Leishmania* infection in *R. norvegicus* and to identify the parasite as belonging to the *L. braziliensis* complex. The results showed that the *L. braziliensis* species is present in the urban area of Belo Horizonte, where human cases of cutaneous leishmaniasis have been reported.

The gold standard for identification of *Leishmania* species is isoenzyme analysis, which requires the isolation of parasites in culture and has a low level of sensitivity (Noyes et al., 1998; Medeiros et al., 2002). Attempts to isolate the parasites in culture medium were unsuccessful (data not shown), mainly due to the high degree of fungal contamination that occurred in spite of the steps that were taken to minimize it. The sensitivity of direct observation techniques, such as imprint slides and tissue culture isolation, is directly proportional to the parasite load in the samples, demonstrating the need for more sensitive techniques, such as PCR, to detect infection (Dias et al., 1977; Falqueto et al., 1986; Oliveira et al., 2005; Fagundes et al., 2010).

The frequency of positive animals was 36.25% higher than what has been reported in other studies. For example, Brandão-Filho et al. (2003) detected *Leishmania* in 18.7% of the spleens from wild and synanthropic rodents in Amaraji (Pernambuco state in Brazil) and Oliveira et al. (2005) reported infections in 12% of skin and blood samples from wild and synanthropic rodents in the city of Araçuaí (Minas Gerais state). A previous study conducted in the northeast district of Belo Horizonte reported that two specimens of *R. norvegicus* that were captured in a peridomestic environment were infected with *Leishmania*, according to PCR directed toward kDNA (Ferreira et al., 2010). Differences in the infection rate compared to the results of this study may be attributable to, among other factors, the study site, the time at which the animals were captured, the rodent species, the number of tissues that were evaluated and the target chosen for PCR. In this study, the presence of the parasite in different tissues indicates its localization in the body, for example, in the blood and skin, with visceralization indicated by its detection in the bone marrow and spleen. Similar results have also been reported by others (Nery-Guimarães et al., 1968; Lainson et al., 1981; Roque et al., 2010).

The highest correlation was observed between blood and bone marrow tissues, which showed the highest rates of infection. In line with these findings, Oliveira et al. (2005) observed a greater sensitivity in PCR assays that detect *Leishmania* spp. in blood samples collected on filter paper, compared to those performed on the skin debris of rodents.

The environments where *Leishmania*-positive animals were collected from breeding sites are beneficial to both the

proliferation of *R. norvegicus* and the occurrence of *Leishmania* transmission cycles. In the Venda Nova and North districts, notable features include the intense accumulation of garbage, open sewers near houses, a dense population and the presence of dogs. The Pampulha district, despite being considered a wealthy area of the city, has some areas with poor sanitation and a population with a low socio-economic status near the areas selected for rodent capture. The environment of the Lagoa da Pampulha (Pampulha district) is the most diverse. In this region, there is a heavy flow of people, dogs and other animals, such as birds and capybaras. *R. norvegicus* can be seen around the periphery, especially at dusk, coincident with the period when fly vectors are most active.

The results presented here do not allow us to confirm that these rodents serve as secondary reservoirs of *L. braziliensis* in the areas studied. However, it does not exclude them from acting as circumstantial reservoirs for *L. braziliensis* in some areas of Belo Horizonte. The ability of *R. norvegicus* to be infected with dermatotropic *Leishmania* species was reported by Giannini (1985). Experimental infection of rodents, six with *L. major* and nine with *L. donovani*, revealed that only those infected with *L. major* were able to maintain the infection for long periods of time. Motazedian et al. (2010) reported an infection rate of 52% (30/57) in *R. norvegicus* by *L. major* in Iran, as detected by PCR, and observed the presence of parasites in various tissues, such as the skin of the ear, the liver and the spleen. In support of these findings, serological surveys confirm the infection of rodents with *Leishmania* spp. using different techniques (Azab et al., 1984; Morsy et al., 1994; Di Bella et al., 2003; Psaroulaki et al., 2010). In Greece, Papadogiannakis et al. (2009) identified *L. infantum* in 3.3% (1/16) of *R. norvegicus* specimens by PCR and sequencing. In this case, the identification of the *Leishmania* species was only possible when nested PCR was utilized. These results led the authors to infer that the animal had a low parasite load and a possible resistance to viscerotropic species. This study has identified the infection of *R. norvegicus* by *L. braziliensis* in an area where both visceral and cutaneous leishmaniasis occur, suggesting that *R. norvegicus* and other rodents are more closely related to the cycle of the dermatotropic species.

Some of the qualities that are necessary for an animal to serve as a reservoir, according to Ashford (1996), are observed in *R. norvegicus*. They are an aggregated species, are sufficiently long-lived to maintain the infectious agent (with an average lifespan of 24 months) and are asymptomatic. Other important factors include the presence of the vector species, *Lu. whitmani* and *Lu. intermedia*, in the city of Belo Horizonte (Souza et al., 2004; Saraiva et al., 2010), the high rate of infected animals harboring *L. braziliensis*, the same species that has been found in human CL cases in the area studied (Passos et al., 1999), and parasitism of the blood and skin of animals, which are the routes of infection for the vector.

The ability of infected rodents to serve as sources of infection for fly vectors and the genetic variability of the parasite involved in the infection of these vectors for humans and rodents need to be characterized. Research is needed to fill the gaps in knowledge regarding the par-

ticipation of *R. norvegicus* in the transmission cycle of leishmaniasis and to clarify the behavior of the disease in the urban context, where the environment is constantly being modified.

The maintenance of rodent control programs and health education are important measures, not only because of the zoonotic potential of these animals for leishmaniasis but also for that of other diseases, such as leptospirosis, which are linked to outbreaks in urban environments.

### Conflict of interest statement

The authors declare that they have no competing interests.

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