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Notes and Comments

Molecular diagnosis of *Leishmania infantum* in wildlife from Botucatu municipality, São Paulo state, Brazil

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Visceral leishmaniasis (VL) is one the most important zoonotic diseases worldwide. Brazil has one of the highest incidences of VL, with > 2000 new cases reported annually and a lethality rate of approximately 8% (SINAN, 2024). The protozoan *Leishmania infantum* (Nicolle, 1908) is the etiologic agent of VL in Brazil and the hematophagous sandfly *Lutzomyia longipalpis* (Lutz & Neiva, 1912) is the main vector. Controlling this disease remains extremely challenging and the current strategies implemented by the Brazilian Ministry of Health are ineffective in reducing the prevalence of infection among dogs, which are the main urban reservoirs of the parasite. The geographical distribution of VL in Brazil is continuously expanding (CVE, 2025).

São Paulo state (SP), the most populated and developed state in Brazil, has experienced a rapid spread of VL since the first canine autochthonous case in 1998 in the Araçatuba municipality (Camargo-Neves et al., 2001; Sevá et al., 2017). The following years were characterized by a geographical dispersion of VL, with its main vector being towards the southeast of SP. The expansion of VL to nonendemic areas of SP has been associated with factors such as the construction of the Bolivia–Brazil gas pipeline and its proximity to the Marechal Rondon highway. In the last years, the distribution of VL in SP mostly comprises the northwestern region of the state (Figure 1), with predictions of its geographical expansion to other localities, including the central region of SP (Sevá et al., 2017).

Although dogs are the most important hosts for VL, infection by *L. infantum* has been described in several wild and domestic animal species in Brazil. The role of wild mammals as hosts of *Leishmania* is complex, and studies on long-lasting infections in these animals are scarce (Roque and Jansen, 2014). Nonetheless, wildlife diagnoses can provide valuable epidemiological data regarding the circulation of *L. infantum* in a given region, thus contributing to the surveillance of VL (Caldart et al., 2021).

Botucatu is a mid-sized city located in the central region of SP, which is the main dispersion route of VL in this state (Figure 1). Some municipalities located <100 km from Botucatu have been long considered highly endemic for VL, compelling public health authorities to monitor the disease in dogs and the vector in urban areas. Previous surveillance studies found no serological evidence of VL among dogs (Babboni et al., 2015) or the presence of *L. longipalpis* (Cutolo et al., 2013) in Botucatu. According to the latest records (June 2025) of the SP Epidemiological Surveillance Center, SP is classified as a non-receptive, silent, and vulnerable area for VL, with no records of autochthonous cases of VL (humans or dogs) (CVE, 2025). However, *L. longipalpis* was first detect in April 2025, which will likely change this classification (Prefeitura de Botucatu, 2025).

Between 2007 and 2013, our research group performed a serological investigation for *L. infantum* in 528 free-ranging wild mammals to contribute to the surveillance of VL in Botucatu (Paiz et al., 2015). Surprisingly, a 1.7% seroprevalence was detected, indicating natural exposure to the parasite. These findings led us to conceive the present study, wherein we further investigated the wildlife from Botucatu (including 280 animals from our previous serological study) to confirm the presence of *L. infantum* in this region. Thus, the objective of this study was to perform the molecular diagnosis of *L. infantum* in wildlife from Botucatu.

Blood samples from 311 free-ranging wild mammals were initially selected for molecular diagnosis of *L. infantum*. These consisted of opportunistic samples obtained during previous studies on leptospirosis (Fornazari et al., 2018) and brucellosis (Batista et al., 2019) in wildlife, collected

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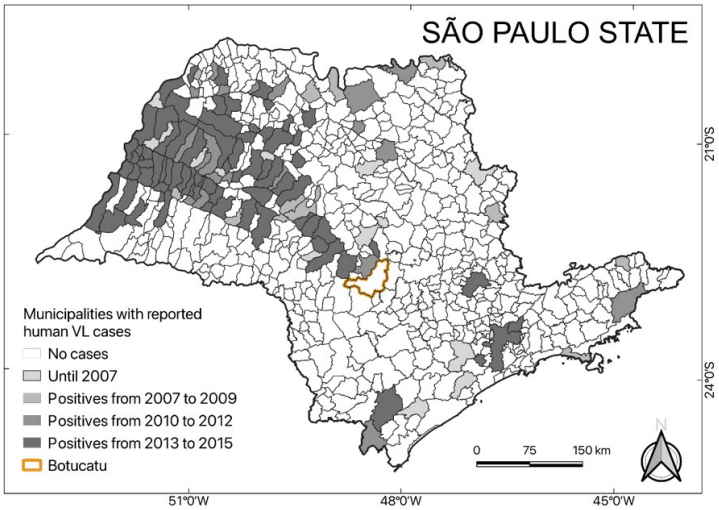

Figure 1. Municipalities of São Paulo state, Brazil, with cases of human visceral leishmaniasis. Botucatu municipality (orange outline) is located in the central region of the state, in the dispersion route direction of visceral leishmaniasis.

between 2012 and 2015, in urban and rural areas of Botucatu or surrounding municipalities. Two methods were used simultaneously to sample the animals: (1) capture in their natural habitat using tomahawk traps and (2) following the daily routine of the Center for Medicine and Wildlife Research (CEMPAS), School of Veterinary Medicine and Animal Science (FMVZ), São Paulo State University (UNESP), Botucatu, SP. Details of the sampling methods have been described previously (Fornazari et al., 2018). The procedures were approved by the Chico Mendes Institute for Biodiversity Conservation (ICMBio, license no 33162-2) and the Ethics Committee of the FMVZ (CEUA Protocol no. 12/2012).

DNA was extracted from blood samples using the Illustra™ Blood Genomic Prep Mini Spin kit (Cytiva, Marlborough, MA, USA) in accordance with the manufacturer's instructions. The final volume of eluted DNA of each sample was 100 µL. Before the molecular diagnosis of *L. infantum*, the samples were submitted to polymerase chain reaction (PCR) targeting the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is present in the mammal genome. This assay was used to assess the effectiveness of the DNA extraction and the presence of PCR inhibitors. Reactions were conducted in sterile DNAse/RNAse-free microtubes (0.2 mL) containing 7.5 µL of ultra-pure water, 1 µL of each oligonucleotide (10 µM), 12.5 µL of GoTaq® Green Master Mix (Promega, Madison, WI, USA), and 3 µL of sample. The microtubes were subjected to denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Amplification was performed in an Eppendorf Mastercycle™ PRO (Eppendorf, Hamburg, Germany) thermocycler and the products were visualized by horizontal electrophoresis using an agarose gel (1.5%) stained with Nancy-520 (MilliporeSigma, Burlington, MA, USA). Samples with an expected size of 399 bp were considered positive and included in the diagnosis of *L. infantum*, whereas negative samples were excluded from the study.

L. infantum was detected using real-time PCR (qPCR-Leish), targeting a conserved region of the minicircle kinetoplast DNA of *Leishmania* parasites (Passos et al., 1996). Reactions were conducted in sterile DNAse/RNAse-free microplates (96 wells), with each well containing 3.8 µL of ultra-pure water, 0.1 µL of each oligonucleotide (10 µM), 5 µL of SYBR®Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µL of sample. The microplates were sealed and subjected to the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification (95 °C for 40 s, 55 °C for 30 s, and 72 °C for 10 s) and melting curve analysis (60 to 95 °C for 55 min). Amplification was performed using the StepOne™ Plus Real-Time PCR System (Thermo Fisher Scientific) and the sizes of the DNA products were compared using melting curve analysis. The positive controls included one sample of *L. infantum* maintained in Novy-MacNeal-Nicolle/liver infusion tryptose medium and two blood samples from naturally infected dogs in an endemic area (Bauru municipality, SP; 96 km from Botucatu) that were seropositive for VL. Ultrapure water was used as the negative control. All the control samples were subjected to DNA extraction using a previously described protocol. The mean melting temperature (Tm) of positive controls was 79.2 °C, and samples with Tm ranging from 78.2 to 80.2 °C were considered positive. All samples were processed in duplicate.

Among the 311 samples initially screened for the GAPDH, two were negative and were excluded from the study. Thus, the overall sample size was 309 animals distributed across 18 species (Table 1). Most of the animals belonged to the species white-eared opossum (*Didelphis albiventris*; n=193) (Lund, 1840) and coati (*Nasua nasua*; n=61) (Linnaeus, 1766). Most of the samples (90.6%, 280) were from Botucatu, and only a few (19.4%, 29) were from 15 nearby municipalities. Among the 309 animals tested, 208 had serological results from our previous study (Paiz et al., 2015). All samples tested negative through qPCR-Leish.

Table 1. Species and number of wild animals tested for *Leishmania infantum* infection in the central region of São Paulo state, Brazil.

Species	Number
Brown howler (*Alouatta guariba*)	1
Crab-eating fox (*Cerdocyon thous*)	3
Azara's agouti (*Dasyprocta azarae*)	1
Lesser grison (*Galictis cuja*)	5
White-eared opossum (*Didelphis albiventris*)	193
Taira (*Eira barbara*)	2
European hare (*Lepus europaeus*)	2
Maned wolf (*Chrysocyon brachyurus*)	1
Capuchin monkey (*Sapajus nigritus*)	2
Crab-eating racoon (*Procyon cancrivorus*)	1
Puma (*Puma concolor*)	2
Orange-spined hairy dwarf porcupine (*Sphiggurus villosus*)	14
Coati (*Nasua nasua*)	61
Hoary fox (*Lycalopex vetulus*)	2
Giant anteater (*Myrmecophaga tridactyla*)	6
Southern tamandua (*Tamandua tetradactyla*)	5
Nine-banded armadillo (*Dasypus novemcinctus*)	4
Gray brocket (*Subulo gouazoubira*)	4
Total	**309**

These results indicated that wildlife from Botucatu was not infected with *L. infantum*. These findings are unsurprising, as no autochthonous cases of VL had been described among dogs and humans by June 2025 (CVE, 2025). We considered two main hypotheses to explain the previously reported 1.7% seroprevalence. First, the serological method employed (Paiz et al., 2015) was the direct agglutination test (DAT), which has never been validated in multiple wild species. Cross-reactivity between phylogenetically close pathogens may occur during serological tests, and it is possible that these seropositive animals were exposed to other trypanosomatid parasites. An alternative explanation is that *L. infantum* occurs at a very low prevalence in wildlife in Botucatu, and none of the studied animals were infected. Serological tests often result in higher rates of positive animals than molecular tests, and because DAT revealed 1.7% seropositivity, one should expect a 0% prevalence using PCR tests. In addition, blood may not be the most suitable type of sample to detect *L. infantum*. In general, parasites belonging to the genus *Leishmania* exhibit a pronounced tropism for macrophages (Naderer and McConville, 2008), and samples from the bone marrow and lymph nodes may yield the highest number of positive results (Travi et al., 2018). However, the use of tissue samples requires more laborious procedures involving animals and blood is easier to obtain, facilitating the assessment of a large sample size.

Despite the difficulties, we recommend the use of tissue samples for future investigations involving the surveillance of VL in wildlife. The number of animals tested in the present study is among the largest ever investigated for *L. infantum* in wildlife.

In conclusion, infection by *L. infantum* was not detected in the wildlife from Botucatu, despite serological evidence from a few animals. These results corroborate the absence of VL cases in this municipality, indicating that *L. infantum* does not occur in the studied area.

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Data Availability Statement

The research data are only available upon request to the corresponding author.

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