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Detection of *Leishmania infantum* DNA in blood samples of horses (*Equus caballus*) and donkeys (*Equus asinus*) by PCR

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ABSTRACT

Visceral leishmaniasis (VL) is a neglected tropical disease caused by the *Leishmania infantum* parasite. The protozoan is able to infect several domestic and wild mammals. Since the first report on *Leishmania* spp. infection in horses in South America, leishmaniasis in equids has been highlighted in Brazil. A molecular epidemiological survey was carried out to verify the occurrence of *Leishmania* spp. DNA in horses and donkeys, in leishmaniases endemic areas in Sao Paulo State, Brazil. To this end, blood samples were obtained from 107 horses and 36 donkeys and subjected to DNA extraction followed by PCR targeting the ITS-1 region. Among the horses and donkeys, 1.87% (2/107) and 8.33% (3/36) were positive by PCR, respectively. The DNA sequencing of the ITS-1 amplification products confirmed *L. infantum* DNA in these animals. Our results suggest that horses and donkeys from non-VL and VL endemic areas of São Paulo State may be infected by the parasite.

KEYWORDS: Asinines. Equines. Equids. *Leishmania infantum.* ITS-1. PCR.

INTRODUCTION

Leishmaniases are vector-borne infectious diseases that can manifest in several mammals, including humans[1]. They are caused by protozoans of the genus *Leishmania* and are transmitted by different genera of Phlebotomine sandflies[2]. The diseases are endemic in several countries, including Brazil, which presents the majority of cases in South America, and is the only country that has a high burden of the two clinical forms of disease[1]. Cutaneous (CL) and visceral leishmaniasis (VL) are the main manifestations of the disease in humans, according to *Leishmania* species infection[2].

Horses infected by *L. braziliensis* were first described in South America in 1927[3]. Alencar, in 1959, described the first infection of the same parasite in donkeys from Brazil[4]. Since then, cases of *L. braziliensis* infecting horses and donkeys have been described[4-13], with some authors suggesting their participation as primary reservoirs in the CL transmission cycle[10,11,13].

Although dogs are considered the main *L. infantum* reservoir in the zoonotic VL cycle, some studies have shown that other mammals, such as cats and horses, can be infected by the parasite, developing symptomatic disease or remaining asymptomatic[14,15]. In fact, leishmaniasis in equids caused by *L. infantum* has also been confirmed in European countries[16,17]. In South America, *L. infantum* infection in horses was reported for the first time in two animals from Brazil, with skin

lesions and locomotor problems[14], followed by Benassi *et al*.[18] who described the first case of *L. infantum* infection in two asymptomatic horses in Sao Paulo State. To date, the epidemiological magnitude and impact of *L. infantum* infection in equids remains uncertain.

The diagnosis of *Leishmania* spp. infection in equids is generally based on parasitological, serological or molecular approaches[19,20]. The microscopic observation of the parasite within macrophages in tissue smears stained by Giemsa is considered the gold standard for the diagnosis[19,20]. However, the low sensitivity of direct microscopy is the main limitation of this technique[10]. Serological tests, such as the immunofluorescence antibody test (IFAT), immunoenzymatic assays (ELISA), and the direct agglutination test (DAT) are often used to detect anti-*Leishmania* spp. antibodies in equids[14,17-20]. Nevertheless, deficiencies regarding sensitivity (due to low antibody levels) and specificity (due to cross-reactions between *Leishmania* spp. and other trypanosomatids parasites) have also been reported[20]. In this sense, molecular techniques such as PCR followed by sequencing, help on diagnosis confirmation and identification of infectious *Leishmania* species in equids[14,17,18,20].

Taking into account all these facts, the aim of this study was to perform an epidemiological molecular survey to verify the occurrence of *Leishmania* spp. DNA in horses and donkeys, from endemic areas of both, CL and VL, in Sao Paulo State, Brazil.

MATERIAL AND METHODS

Study area and sample collection

This study was carried out in the equid population of Pirassununga city (CL endemic area) and Jau city (CL and VL endemic area), both counties of Sao Paulo State, Brazil. A convenience samples was composed of 107 horses from Pirassununga and 36 donkeys from Jau, between 2016 and 2017. Approximately 5 mL of blood were collected from the jugular vein of the animals, and thereafter they were submitted to DNA extraction. No clinical assessment was performed in these animals.

Ethical issues

The study was approved by the Ethics Committee for the Use of Animals at the Faculty of Animal Science and Food Engineering of the University of Sao Paulo (under the process Nº 3615240516 and 8335160218) and was performed in compliance with national guidelines. All horse owners consented to have their animals sampled.

DNA extraction

DNA extraction from blood samples was performed using the Illustra™ Blood Genomic Prep Mini Spin kit (GE Healthcare Life Sciences, Uppsala, Sweden), according to the manufacturer's recommendations. The DNA samples were stored at -20 °C until the amplification.

Endogenous gene control

To exclude false negatives due to DNA sample degradation, real-time PCR for the endogenous β-actin gene was performed according to Manna *et al*.[21] with the primers F-5'-dCTGGCACCACACCTTCTACAA-3' and R-5'-dGCCTCGGTCAGCAGCA-3' and the hydrolysis probe 5'-CCACGCGCAGCTCG-3' following a previous protocol[21]. Amplification was performed using a LightCycler® 480 II thermocycler (Roche, Rotkreuz, Switzerland). The standard reaction curve was obtained using canine DNA in a ten-fold serial dilution. The DNA concentration was estimated by measuring the absorbance at 260 and 280 nm in a DS-11® spectrophotometer (DeNovix Inc. Wilmington, DE, USA). Sterilized ultrapure water was used as the negative control.

Leishmania spp. DNA amplification and sequencing

Samples were tested using a conventional PCR targeting the *Leishmania* spp. internal transcribed spacer 1 (ITS-1) region of a ribosomal DNA (rDNA)[22] with primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5-8S (5'-TGATACCACTTATCGCACTT-3'), generating a DNA fragment of 300 to 350 base pairs (bp) depending on the *Leishmania* species. The reaction mixtures consisted of 1 U of Platinum® Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 14.65 µL of ultrapure water, 1X of PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs (dATP, dCTP, dGTP, and dTTP), 12.5 pmol of each primer, and 2.5 µL of extracted DNA from blood. The thermal cycling conditions consisted of 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 s, 53 °C for 30 s, 72 °C for 1 s, and 72 °C for 5 min. The DNA sample of *L. amazonensis* (IFLA/BR/1967/ph8) provided by the Leishmaniasis Laboratory of the Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, and sterilized ultrapure water were used as positive and negative controls, respectively.

After the amplification product detection through electrophoresis on a 1.5% agarose gel, the PCR products were excised from the gel and purified using the Illustra™ GFX PCR DNA and Gel Band Purification Kit (GE

Healthcare Life Sciences, Little Chalfont, UK), according to the manufacturer's instructions. DNA sequencing was performed using 20 ng/μL of purified PCR products and 5 μM of each primer at the DNA Sequencing Service of the Human Genome and Stem Cell Research Center (HUG-CELL), Institute of Biology (IB), University of Sao Paulo (USP), Sao Paulo, Brazil.

Chromatograms obtained with the forward and reverse primers were assembled in the Sequence Scanner Software version 2.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) for their integrity. Next, the sequences were aligned in the Clustal W software (available in BioEdit Sequence Alignment Editor software, version 7.1.11, Ibis Biosciences, Carlsbad, CA, USA). The obtained consensus sequence was subjected to the Basic Local Alignment Search Tool (BLAST) for the alignment with sequences available in the GenBank database. Species identification of *Leishmania* spp. was considered correct when the sequences showed over 98% identity for at least 99% of the analyzed sequence.

RESULTS

All samples in this study were positive for the endogenous β-actin gene, confirming the quality of DNA extraction protocol. Regarding molecular analyses, 1.87% (2/107) of the horses and 8.33% (3/36) of the donkeys were PCR-positive for the amplified ITS-1 region of *Leishmania* spp. (Figure 1, Table 1) and submitted to DNA sequencing (Table 2).

The sequencing and BLAST search in the GenBank database showed two horses with ≥ 98,56% identity with *L. infantum* ITS-1 sequences (similarity with sequences MN412822.1 and MN648767.1) (Table 2). Regarding the donkeys, sequences from three animals revealed 100% identity with *L. infantum* ITS-1 sequences (similarity with sequences MN648768.1 and MN648764.1) (Table 2).

DISCUSSION

Since the first report on *Leishmania* spp. infection in

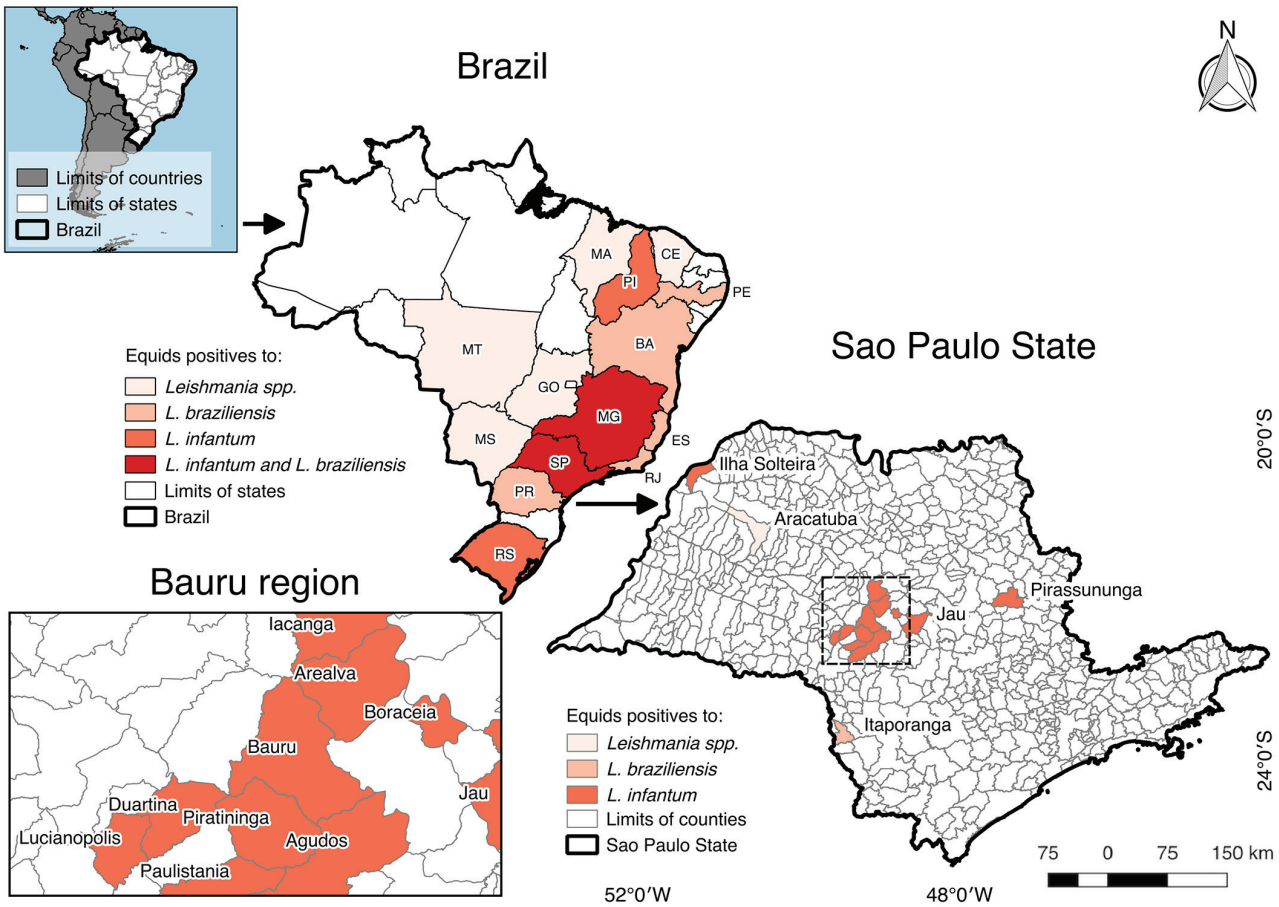

Figure 1 - Equids that are positive to *Leishmania* spp., described by epidemiological surveys from Brazil and Sao Paulo State. Illustrative map based on twenty articles published in peer reviewed scientific journals between 1959 and 2019, and results from the present study.

Table 1 - Epidemiological survey studies on leishmaniasis in equids in counties of Sao Paulo State, Brazil.

References	Municipality	Animals	Clinical signs	IFAT (%)	ELISA (%)	PCR (DNA target) (%)	Parasitological detection*	Isolation	Sequencing (% identity)
Yoshida *et al.*[12]	Itaporanga	1	Present	-	-	-	100%	*L. braziliensis*†	-
Villalobos *et al.*[24]	Agudos	16	-	12.50%	-	-	-	-	-
	Arealva	6	-	33.33%	-	-	-	-	-
	Bauru	30	-	46.67%	-	-	-	-	-
	Boraceia	6	-	50.0%	-	-	-	-	-
	Duartina	9	-	22.22%	-	-	-	-	-
	Iacanga	2		50.0%	-	-			
	Lucianopolis	10	-	50.0%	-	-	-	-	-
	Paulistania	6	-	50.0%	-	-	-	-	-
	Piratininga	15	-	53.33%	-	-	-	-	-
Feitosa *et al.*[25]	Araçatuba	466	-	-	14.59%	-	-	-	-
Benassi *et al.*[18]	Ilha Solteira	40	Absence	2.50%	-	15% (*L. infantum*)	-	-	99.3% *L. infantum*
In this study	Pirassununga	107	-	-	-	1.87% (*Leishmania* spp.)	-	-	≥ 98,5% *L. infantum*
	Jau	36	-	-	-	8.33% (*Leishmania* spp.)	-	-	100% *L. infantum*

*Visualization by microscopy from cytology, histology or imprint of lesions; †based on zymodeme and serodeme analysis

horses in South America, some epidemiological surveys have shown positive equids to *Leishmania* spp. by parasitological, molecular and serological methods in Brazil (Figure 1)[4-12,14,18,23-31].

Regarding Sao Paulo State, Yoshida *et al.*[12] reported an equine infected by *L. braziliensis* in a CL endemic area of the State (Itaporanga city) (Figure 1, Table 1). Additionally, in Bauru city region, where CL and VL are endemic, 40% of tested animals were seropositive for *L. infantum* (Figure 1, Table 1)[24]. According to Feitosa *et al.*[25], 14.59% of the equines sampled presented antibodies against *Leishmania* spp. in other VL endemic areas of the State (Figure 1, Table 1). The first case of *L. infantum* DNA detection in horses in Sao Paulo State was reported by Benassi *et al.*[18] in Ilha Solteira city, which is also a VL endemic area (Figure 1, Table 1). Herein, we performed an epidemiological molecular survey in horses from Pirassununga (CL endemic area) and in donkeys from Jau (CL and VL endemic areas), both cities located in Sao Paulo State, Brazil. In both cities, positive animals to *L. infantum* DNA by PCR were found (Figure 1, Table 1, Table 2).

The best interpretation of these findings is that equids' populations of these leishmaniases endemic areas are in close contact with the parasite. Contact favored by the opportunistic feeding habits of *Lutzomyia longipalpis* (VL competent vector), which can feed on a wide variety of vertebrates[32]. Studies on the feed blood source of *Lu. longipalpis* showed that horses and donkeys can be used as a blood source[32,33]. According to Oliveira-Pereira *et al.*[34], horses were not a preferred source of blood but were frequently baited by Phlebotomine sandflies, suggesting the need to investigate this species as a possible reservoir.

To the best of our knowledge, this is the first report on the finding of *L. infantum* DNA in donkeys blood samples in Brazil. These animals are from Jau, an important VL endemic area of Sao Paulo State (Figure 1). This finding confirms that equids are exposed to and may be infected by *L. infantum* in VL endemic areas[35], since they usually live in contact with infected VL vector[36].

However, despite being an important CL endemic area, VL is not endemic in Pirassununga. In this city, we found two horses with *L. infantum* DNA in blood samples (Figure 1, Table 1, Table 2). It is important to highlight that we cannot exclude the possibility that these animals could have been imported from other regions where VL is endemic. Particularly, the intense movement of equids between regions of different endemicities could be a risk factor for the introduction of leishmaniasis in non-endemic areas[20,33]. One cat and one cattle that were positive to *L. infantum* by PCR were also reported in this county[15,37].

Table 2 - *Leishmania* species identification from PCR-positive blood samples, by sequencing of ITS-1 rDNA.

Animal	Species	Consensus	Size*	Query cover	Identity	BLAST search (GenBank sequence similarity)
1	Donkey	>Consensus 1 CAGTCATCCATCGCGACACGTTATGTGAGCCGTTAT CCACACACGCACCCACCCCGCCAAAAACCGAAAC GCCGTATATTTTTTGTATAAACGGACATTTT	101 bp	99%	100%	*L. infantum* (MN648768.1)
2	Donkey	>Consensus 2 TCTGGATCATTTTCCGATGATTACACCCAAAAAAC ATATACAACTCGGGGAGACCTATGTATATATATGTAGG CCTTTCCCACATACACAGCAAAGTTTTGTACTCAAAA TTTGCAGTAAAAAAAAGGCCGATCGACGTTATAACG CACCGCCTATACAAAAGCAAAAATGTCCGTTTATACA AAAAATATACGGCGTTTCGGTTTTTGGCGGGGTGGG TGCGTGTGTGGATAACGGCTCACATAACGTGTCGCG ATGGATGAC	264 bp	100%	100%	*L. infantum* (MN648764.1)
3	Donkey	>Consensus 3 TCTGGATCATTTTCCGATGATTACACCCAAAAAACAT ATACAACTCGGGGAGACCTATGTATATATATGTAGGC CTTTCCCACATACACAGCAAAGTTTTGTACTCAAAA TTTGCAGTAAAAAAAAGGCCGATCGACGTTATAACG CACCGCCTATACAAAAGCAAAAATGTCCGTTTATAC AAAAAATATACGGCGTTTCGGTTTTTGGCGGGGT GGGTGCGTGTGTGGATAACGGCTCACATAACGTG TCGCGATGGATGAC	264 bp	100%	100%	*L. infantum* (MN648764.1)
4	Horse	>Consensus 4 TCTGGATCATTTTCCGATGATTACACCCAAAAAAC ATATACAACTCGGGGAGACCTATGTATATATATGTAG GCCTTTCCCACATACACAGCAAAGTTTTGTACTC AAAATTTGCAGTAAAAAAAAGGCCGATCGACGTT ATAACGCACCGCCTATACAAAAGCAAAATGTCCG TTTATACAAAAAATATACGGCGTTTCGGTTTTTGG CGGGGTGGGTGCGTGTGTGGATAACGGCTCAC ATAACGTGTCGCGATGGATGAC	263 bp	100%	99.62%	*L. infantum* (MN412822.1)
5	Horse	>Consensus 5 ATGTATATATATGTAGGCCTTTCCCACATACACAGC AAAGTTTTGTACTCAAAATTTGCAGTAAAAAAAAG GCCRATCGACGTTATAMCGCACCSCCTATACAAA AGCAAAAATGTCCGTTTATACAAAAAATATACGGC GTTTCGGTTTTTGGCGGGGTGGGTGCGTGTGT GGATAACGGCTCACATAACGTGTCGCGATGGATGAC	208 bp	100%	98.56%	*L. infantum* (MN648767.1)

*in base pairs (bp)

Somehow, more epidemiological surveys on the equids' population of the county together with entomological surveys are fundamental for measuring the magnitude of these findings in this non-VL endemic region.

It is important to consider that *L. infantum* DNA found in the blood of equids does not imply them as VL reservoirs but suggests that they may be accidental hosts[26,38]. When donkeys were challenged with promastigotes of *L. infantum* and followed-up for 12 months, Cerqueira *et al*.[39] concluded that donkeys were able to overcome the experimental *Leishmania* infection and did not infect *Lu. longipalpis* vector under laboratory conditions. Consequently, they cannot be considered an important reservoir in the epidemiological chain of transmission of *L. infantum*, although they represent an important blood source for the vector and their proliferation. Nevertheless, no other study regarding xenodiagnosis in equids has been performed so far. In addition, the natural role of equids in the leishmaniases transmission has not yet been demonstrated or refuted[19,29], and the impact of these animals on leishmaniases epidemiological cycles remains unclear[20].

Hence, we recognize the urgency and importance of investigating the equids' populations to improve the understanding of the epidemiology of *L. infantum* infection in these animals[19,29]. *L. infantum* DNA detection in the blood samples of horses and donkeys suggests that these

animals can be infected by the parasite in the studied areas. In addition, our results suggest that *L. infantum* potentially circulates among equids from VL and non-VL endemic areas of Sao Paulo State, Brazil.

CONCLUSION

L. infantum DNA detection in blood samples of horses and donkeys by PCR in non-VL and VL endemic areas of Sao Paulo State indicates that these animals can be infected by the parasite.

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AUTHORS' CONTRIBUTIONS

Conceptualization: JAFL, BT, TMFSO; data curation: BT, JAFL; formal analysis: JAFL, RMS, BT, TMFSO; funding acquisition: BT, TMFSO; investigation: BT, JAA, JCB, RAB; methodology: BT, JAA, JAFL, JCB, RAB, HLF, LKB; project administration: TMFSO; software: JAFL, RMS, HLF, LKB; supervision: HLF, LKB, RAB, TMFSO; writing-original draft: JAFL, GV, TMFSO; writing-review & editing: JAFL, GV, TMFSO, RMS, LKB, HLF, RAB.

ETHICAL STATEMENT

The present study was approved by the Ethics Committee for the Use of Animals at the Faculty of Animal Science and Food Engineering of the University of Sao Paulo (under the processes Nº 3615240516 and 8335160218) and was performed in compliance with national guidelines. All horse owners consented to have their animals sampled.

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