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Short Communication

Molecular detection of *Leishmania* spp. in cattle from Brazil by means of PCR using internal transcribed spacer 1

Detecção molecular de *Leishmania* spp. em gado no Brasil por PCR com espaçador interno transcrito 1

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Abstract

Leishmania spp. are important agents of human and animal leishmaniases that have an important impact on public health. In this study, we aimed to detect the circulation of *Leishmania* spp. in cattle from a visceral leishmaniasis non-endemic area of the state of São Paulo, Brazil. DNA was extracted from blood samples from 100 heifers in the municipality of Pirassununga and was amplified using primers specific for the first internal transcriber spacer (ITS1), to assess the presence of trypanosomatids. The assays revealed that one sample presented bands of between 300 and 350 base pairs. In GenBank, this sample matched 100% with *Leishmania infantum* (314 base pairs). The results suggest that cattle can be infected by *Leishmania infantum* in Brazil.

Keywords: Cattle, ITS1, *Leishmania infantum*, São Paulo.

Resumo

Leishmania spp. são agentes causadores das leishmanioses em humanos e em animais, gerando grande impacto à saúde pública. Este estudo objetivou detectar a circulação de *Leishmania* spp. em área não endêmica para leishmaniose visceral de São Paulo, Brasil. Foram extraídas amostras de DNA de 100 novilhas da cidade de Pirassununga. Estas amostras foram amplificadas com os iniciadores específicos para tripanosomatídeos *Internal Transcriber Spacer 1* (ITS1). Os ensaios revelaram uma amostra com bandas entre 300 e 350 pares de base (pb). A amostra demonstrou 100% de identidade com *Leishmania infantum* (314 pb). Os resultados sugerem que o gado pode ser infectado por *L. infantum* no Brasil.

Palavras-chave: Bovinos, ITS1, *Leishmania infantum*, São Paulo.

Leishmania infantum causes human and animal visceral leishmaniasis (VL) in Europe, North Africa and South America (ABRANTES et al., 2016). In Brazil, dogs are its major hosts and are targeted for visceral leishmaniasis control, along with sandflies of *Lutzomyia* sp., the vector for leishmania (BRASIL, 2014). However, a wide range of other possible reservoirs exists (WHO, 2015) including cats (OLIVEIRA et al., 2015; BENASSI et al., 2017) and horses (SOARES et al., 2013). As a result, cases of VL have sharply increased since the 1980s. Furthermore, cases of bovine infection by *Leishmania* spp. have been reported in Switzerland, China and India, while *Leishmania* spp. antibodies have been detected in cattle in Bangladesh and Zimbabwe (DUBEY et al., 1998; LOBSIGER et al., 2010; ALAM et al., 2011; SINGH et al., 2013; GAO et al, 2015). To date, no cases of *L. infantum* infecting cattle have been reported in non-endemic areas of Brazil. In the light of the high social, economic and health burdens brought by leishmaniasis, molecular methods of parasite detection and characterization have been developed to assist in disease treatment and control (DUNCAN, 2014).

The present study was conducted in the municipality of Pirassununga, state of São Paulo, Brazil, where VL does not occur endemically (BRASIL, 2018). Between 2014 and 2015, blood samples were donated from 100 heifers that were born on the Fernando Costa campus of the University of São Paulo (USP) at Pirassununga, São Paulo, Brazil. The samples were stored

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at –20 °C. This study was approved by the university's Ethics Committee for Animal Use (CEUA) under CEUA registration number 4580261017.

DNA extraction from blood samples was performed using a kit for DNA isolation from cells and tissues (Qiagen, USA), in accordance with the manufacturer's recommendations. The DNA thus extracted was stored at –20 °C until analysis. All samples in this study were found to be positive for the endogenous β-actin gene, and this was determined as described by Manna et al. (2006), with analysis as described by Bustin et al. (2009).

PCR amplification for trypanosomatid detection was performed as described by El Tai et al. (2000), using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5-8S (5'-TGATACCACTTATCGCACTT-3'). These target ITS1 rRNA and amplify a segment that varies depending on the species (EL TAI et al., 2000; TENÓRIO et al., 2014). A DNA sample extracted from *L. infantum* (MCAN/BR/1984/CCC-17.481), which was provided by the Leishmaniasis Laboratory at the Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, was used as a positive control. To avoid contamination with the positive control, this sample was the last to be handled in all steps. DNA purification for sequencing was done from a second PCR reaction using a GE Healthcare kit (Illustra® GFX PCR DNA and gel band purification kit), before electrophoresis. The sequences were analyzed at the DNA sequencing service of the Human Genome and Stem Cell Research Center, Biological Institute (IB), USP. Chromatograms obtained with the forward and reverse primers were assembled with the Sequence Scanner Software 2 v2.2. The sequences were manipulated with Clustal W available in the BioEdit Sequence Alignment Editor version 7.1.11 (HALL,1999). The assembled contigs were submitted to BLAST search (ALTSCHUL et al., 1990), and hit sequences were retrieved.

Blood samples donated from 100 heifers from the state of São Paulo, Brazil, were tested by means of PCR directed towards trypanosomatid ITS1. Serological tests could not be performed because we only received red blood cells. Only one sample was PCR-positive, yielding fragments ranging in size from 300 to 350 bp (Figure 1). Direct sequencing and analysis of the amplicons revealed a sequence with 314 bp, which presented 100% matching with the *L. infantum* isolate 135 Hig ITS1 (accession number: MF977315.1).

Although our results only revealed one infected heifer among 100 animals tested, the potential of these animals to serve as *Leishmania* spp. parasite reservoirs is worrisome. Killick-Kendrick (1990) first pointed out that cattle were a potential reservoir for *Leishmania* spp. in India. A later study also pointed towards cattle, along with sheep, goats and donkeys, as the probable source of *L. infantum* that caused a VL outbreak in Jiash, China (GAO et al., 2015). The amplified kDNA from those farm animals matched *Leishmania* spp. isolates from human patients. Lastly, a more recent study revealed that bovine neutrophils and monocyte-derived macrophages (MDM) can be infected by *L. donovani* when co-incubated with its live promastigote (TASEW et al., 2016). Moreover, cattle herds contribute towards the environmental conditions and provide the food source that together allow development of the *Leishmania* spp. vector, i.e. the female sandfly (ROHOUSOVA et al., 2015).

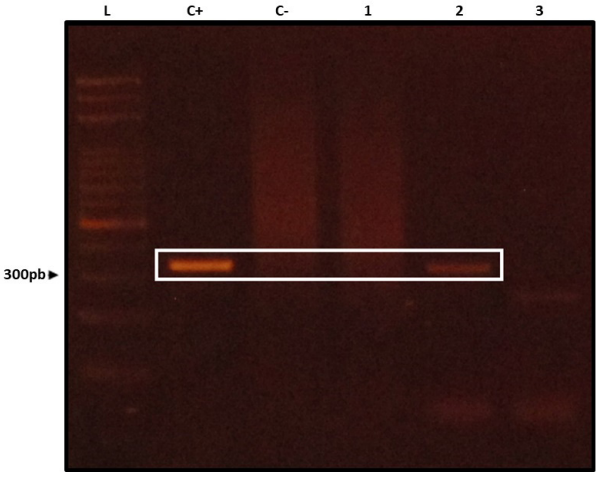

Figure 1. ITS1 blood sample from cattle showing positivity for trypanosomatids. **(L)** 100 bp ladder; **(C+)** DNA of *L. infantum* (MCAN/BR/1984/CCC-17.481) from positive control; **(C-)** negative control; **(1, 3 and 4)** bovine blood samples that were negative for *L. infantum* using ITS1; **(2)** *L. infantum* sample (314 bp) that was positive using ITS1.

Although cattle were found to provide only 0.6% of the blood source for *Lutzomyia* sp. in a rural area in Maranhão, Brazil, it was noted that this was nonetheless a valid blood source, thus providing a reminder that sand flies have opportunistic feeding habits (GUIMARÃES-E-SILVA et al., 2017). On the other hand, a study in Ethiopia showed that cattle were the second most preferred host of *Phlebotomus orientalis* (GEBRESILASSIE et al., 2015). The possibility that presence of cattle might be a risk factor for leishmaniasis in Brazil was discussed by Paixão (2017). However, from spatial analysis, this author concluded that their presence was actually a protective factor against VL in humans. According to this same author, this protective factor may occur due to the large extent of the area where these animals live, in comparison with the limited flight range of *Lutzomyia* spp. Following the same reasoning, our finding may be extremely important in small areas where humans, cattle, other animals and vectors share the same environment. Likewise, the presence of *L. infantum* in cattle also emphasizes the need for active surveillance to detect and diagnose infections in production animals.

No human VL cases have been reported in Pirassununga over the last ten years, but nine cases of cutaneous leishmanisis (CL) were reported over the same period (BRASIL, 2018). Brazil notified 19,402 cases of CL and 3,453 of VL to the World Health Organization in 2014 (WHO, 2016). According to Alvar et al. (2012), underreporting of leishmaniasis in Brazil is considered mild, since the degree of underreporting of VL was found to be between 1.3 and 1.7-fold.

These results suggest that *L. infantum* occurs and potentially circulates among cattle herds in the state of São Paulo, Brazil. Further studies should focus on the prevalence of this infection and on achieving better understanding of the epidemiology of leishmaniasis and the presence of clinical signs and metabolic changes in this animal population. Moreover, entomological and phylogenetical studies are fundamental for understanding the dynamics of transmission of these trypanosomatids in non endemic areas and in their hosts.

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