Parasitology

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First isolation of *Leishmania infantum* by blood culture in bovines from endemic area for canine visceral leishmaniasis

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Research Article

*Identification of *Leishmania infantum* on bovine.

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Abstract

Leishmaniasis is considered a parasitic disease that still causes serious consequences for mankind, because it presents a high mortality rate worldwide. Considered multi-hosts, the parasites of the genus *Leishmania* are able of infecting a wide variety of animal species. The dog was considered the main source of infection of visceral leishmaniasis (VL), in the urban area. However, the role of other animal species in the epidemiological cycle of the disease, such as cattle, remains unclear. Therefore, the aim of the present study was to evaluate the occurrence of *Leishmania* spp. in 100 bovines (*Bos taurus*) from an area endemic for canine VL, using blood culture and molecular analysis. By the sequencing analysis, one sample showed 100% similarity with *Leishmania infantum*. The results provide the first case of *L. infantum* isolation in one bovine from the periurban areas of Bauru, state of São Paulo, Brazil.

Introduction

Leishmaniasis is a neglected tropical disease with a high mortality rate worldwide (Narain *et al.*, 2010; Zhao *et al.*, 2016). The disease can manifest in tegumentary, mucocutaneous or visceral forms (Akilov *et al.*, 2007), and visceral leishmaniasis (VL) is considered to be the most serious, since it is associated with a higher risk of death. The aetiological agents of leishmaniasis are flagellate protozoa belonging to the Trypanosomatidae family, order Kinetoplastida and genus *Leishmania*. In the Americas, *Leishmania* (*Leishmania*) *infantum* is the agent of VL (Brasil, 2014). The disease cycle involves females of sandflies, belonging to the genus *Lutzomyia* as vector, and in the Americas, the main species is *Lutzomyia longipalpis* (Brasil, 2016). When the vector feeds in mammals, it inoculates promastigote forms of the parasite, which after phagocytosis by macrophages, undergo modifications, transforming into amastigote forms that are found in the tissues of the vertebrate host. Brazil accounts for 96% of the human cases of VL reported in the Americas (OPAS, 2017). The municipality of Bauru presents a high incidence rate of VL in humans, being classified as an area of constant canine and human transmission of the disease (Cardim *et al.*, 2016; Brasil, 2016, 2017).

The domestic dog is considered to be the main reservoir of VL, with intense cutaneous parasitism that usually precedes human cases (Reis, 2001; Moshfe *et al.*, 2009). However, several studies have been carried out in an attempt to identify other species of domestic, wild and production animals potentially involved in the epidemiological cycle of VL (Williams *et al.*, 1991; Mukhtar *et al.*, 2000; Zorzetto, 2001; Braga *et al.*, 2014; Carranza-Tamayo *et al.*, 2016). In particular, cattle have been reported as a feeding source of the vector of VL, phlebotomines sandflies, indicating that animals of this species may specifically maintain the parasite in the host population, thereby contributing to the continuity of the life cycle of the disease-causing protozoan (Morrison *et al.*, 1993; Barata *et al.*, 2005; Missawa *et al.*, 2008; Soares *et al.*, 2014).

Euthanasia of seroreagent or parasitologically positive dogs has been implemented as a control measure for VL in endemic areas in Brazil; however, the effectiveness of this measure is uncertain, since other epidemiological factors may be involved in the disease cycle, including the participation of other species (Oliveira *et al.*, 2015; Brasil, 2016; Machado *et al.*, 2016).

Therefore, the aim of the present study was to evaluate the occurrence of *Leishmania* spp. in cattle from an endemic area of canine VL associated with human VL cases, to verify the potential role of cattle in the epidemiological cycle of the disease.

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Material and methods

Study site and blood collection

We collected blood samples from 100 bovines (*Bos taurus*) located in the urban and periurban regions of the municipality of Bauru, São Paulo, Brazil (22°18′54″S, 49°03′39″W). From the caudal vein, 10 mL of blood was collected from each animal. All animals had no clinical signs at the time of blood collection.

Blood culture and DNA extraction

To detect the presence of *L. infantum* in blood samples, the blood culture technique was performed according to the method described by Luz (1999). Each sample of blood was inoculated in three tubes of Liver Infusion Tryptose culture medium, to separate aliquots of 5 mL of plasma, leucocyte layer and red blood cells sediment. All cultures were kept in a B.O.D incubator at 25–26 °C. The samples were observed under an optical microscope (400×) every 2 weeks for a period of 4 months for visual detection of the parasite.

Positive samples determined by the blood culture technique were prepared according to the protocol established by Pinto (2000) for the subsequent extraction of DNA, using the Illustra Blood Genomic Prep Mini Spin Kit (GE Healthcare®), as recommended by the manufacturer.

Polymerase chain reaction

The polymerase chain reactions (PCRs) were performed using 5 μL of PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.0), 1.5 U of Taq polymerase (Platinum®Taq DNA Polymerase, Invitrogen), 10 μM of each oligonucleotide, 1.6 mm $MgCl_2$, 10 mM dNTPs, 2 μL of the test sample and 17.5 μL ultra-pure water (MIX-PCR). The amplification conditions were performed in a thermocycler (Mastercycler®pro-Eppendorf) with initial denaturation at 95 °C for 2 min; followed by 30 cycles at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 1 min; and final extension at 72 °C for 6 min. DNA from *L. infantum* strain MHOM/BR/IOC/2906 was used as a positive control of the reaction and ultra-pure water was used as a negative control. The oligonucleotides used were from the ITS region of the parasite with the following sequence: LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′), described by El Tai *et al.* (2000).

Sequencing

PCR-amplified samples were purified with the ExoSap (GE)-USB ExoSap-IT® enzyme, and then submitted to sequencing (AB I300, Applied Biosystems, Foster City, CA, USA). The DNA sequences were aligned using the ClustalW program by Molecular Evolutionary Genetics Analysis (MEGA version 7.0) for Windows® (Tamura *et al.*, 2011), and submitted to BLAST (http://www.ncbi.nlm.nih.gov/BLAST) for comparison with the sequences deposited in the database.

Results

From the seven samples positive to blood culture, six samples showed similarity with *Trypanosoma theileri*, a species considered non-pathogenic to cattle, which shows a high homology with species of the genus *Leishmania*. However, one sample showed 100% similarity with *L. infantum*. The sequence was deposited in the NCBI database with the accession number, respectively: EF653268.1 (100% identity, query coverage of 47%, and *e*-value of 8e-55).

Discussion

This is the first study to report the occurrence of *L. infantum* in one bovine from the municipality of Bauru, state of São Paulo, Brazil, diagnosed by PCR from blood culture with subsequent sequencing for species confirmation.

Overall, there have been few studies evaluating the role of cattle in the epidemiological cycle of leishmaniasis in Brazil and elsewhere. Most studies have focused on the feeding of the vector of the parasite or on the serological detection of the parasite only (Mukhtar *et al.*, 2000; Singh *et al.*, 2008; Akter *et al.*, 2012). However, molecular techniques are important to determine the participation of different host animals in the cycle of the disease, as well as to identify the parasite species with which host animals are in contact, because the occurrence of other flagellated protozoa in these animals might show cross-reactions when using a serological technique for detection and diagnosis (Verlooo *et al.*, 2000; Luciano *et al.*, 2009; Eyford *et al.*, 2011; Syvagothi *et al.*, 2014; Uzcanga *et al.*, 2016).

Among the few studies that have reported the occurrence of *Leishmania* spp. in cows using molecular techniques, Lobsiger *et al.* (2010) described a case of a cow in Switzerland showing symptoms suggestive of leishmaniasis, and sequencing of the cutaneous tissue sample of the animal revealed 98% sequence similarity with *Leishmania siamensis*. In addition, Bhattarai *et al.* (2010) observed positivity in bovine blood for *Leishmania* spp., using PCR in an area of India with known VL occurrence.

Studies suggest that the presence of cattle in areas of occurrence of leishmaniasis can influence the abundance and infection of the disease vector (Guimarães *et al.*, 2017).

The present study represents the first identification of *L. infantum* in one bovine of an area endemic for VL with both canine and human cases, corroborating with results presented by Gao *et al.* (2015) in a study carried out in China after an outbreak of VL in humans. This study evaluated, among domestic and wild animals, livestock animals, such as cattle, which showed positive Leishmania DNA from PCR, whose DNA sequences of the cattle were coincident with DNA sequences isolated from human patients with VL area of the study, thus suggesting that the evaluated animals may represent reservoir hosts in areas where the disease occurs. This finding emphasizes the need for more studies to evaluate the importance of cattle, and other potential hosts, in the epidemiological context of leishmaniasis. Expansion of the host range will provide new insight into the transmissibility of promastigotes from the phlebotomine vector, and the capacity of different animals to act as reservoirs of this important zoonosis.

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Conflict of interest. None.

Ethical standards. The study was approved by the Ethics Committee on Animal Use (CEUA) of Botucatu Medical School (FMB-UNESP) under approval no. 1095/2014.

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