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Short Communication

Seroprevalence and molecular characterization of *Leishmania* in dogs from an endemic area of zoonotic visceral leishmaniasis in Brazil

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ABSTRACT

Visceral leishmaniasis (VL) can cause large-scale and tenacious epidemics with high fatality rates. Current seroprevalence and circulating *Leishmania* species were evaluated in dogs domiciled in the municipality of Sabará, a small historic and touristic city in the Brazilian state of Minas Gerais. A total of 3926 dogs domiciled in seven different districts of Sabará were serologically tested for canine visceral leishmaniasis (CVL) by indirect enzyme-linked immunosorbent (ELISA) and immunofluorescence (IFA) assays, in a two-years census survey (2011–2012). The average positivity rate of canine infection was 3.4%. Three additional diagnostic tests – imprint/smear direct parasitological, molecular (LnPCR) and myeloculture – were performed in a random sample of fifty seropositive dogs composed of symptomatic (39) and asymptomatic (eleven) animals. LnPCR showed 100% of positivity for *Leishmania* DNA in, at least, one among four tissue samples tested (mesenteric lymph node, skin, spleen and bone marrow), independently of the clinical canine group. Higher and statistically equivalent positivity rates (98% and 96%) for *Leishmania* DNA were found in canine lymph node and spleen. Asymptomatic dogs showed expressive positivity rates in all three additional diagnostic techniques. *Leishmania infantum* was confirmed as the etiological agent of CVL in Sabará.

1. Introduction

Visceral leishmaniasis (VL) can cause large-scale and tenacious epidemics, with high case–fatality rates. In Brazil, rural epidemics were seen in ten-year cycles. Since 1999, a sharp expansion of VL has been observed over the country. VL reached urban areas with increasing numbers of human cases and high prevalence of canine cases [1–4].

The etiological agent of VL in Latin American is *Leishmania infantum*. Human infection occurs after biting of females of *Lutzomyia longipalpis* phlebotomine sand flies previously infected with the parasite [5]. Dogs (*Canis familiaris*) are considered the main domestic reservoirs in the zoonotic cycle of VL [6]; therefore, it is important to know the prevalence of canine VL infection (CVL) in a given place. To achieve that, the reliable identification of infected dogs, even in the absence of typical clinical signs of VL, is critical. A number of parasitological, serological and molecular tests have been developed and comparatively used to diagnose canine *Leishmania* infection [7]. In most cases, the final result will be determined by the clinical state of the dog, parasitemia level, quality of the biological sample and type of method chosen for diagnosis [8].

In the present work, we studied the current infection of dogs by *Leishmania* in a historic and touristic town – named Sabará – in the Brazilian state of Minas Gerais. Sabará is part of the so-called Gold Circuit of Minas Gerais which comprises small towns with historical heritage from the late 1700's. Over the last seven years, human and canine cases of VL have been reported to the Health Department of Sabará. In the last three years, the average number of human cases of VL was higher than 4.4. Hence, the city was classified as area of intense transmission of VL, according to the parameters established by the Brazilian Ministry of Health in the VL Control Program. Aiming at determining the current *Leishmania*

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infection in dogs domiciled in Sabará, we undertook a two-year canine census survey using the standard, antibody-based methods recommended by the Control Program of VL of the Brazilian Ministry of Health [9]. We also compared the performance of three additional diagnostic tests—imprint/smear direct parasitological, molecular (PCR) and myeloculture—in a sample of seropositive dogs and identified the etiological agent of CVL in Sabará at species level.

2. Materials and methods

2.1. Ethical procedures

The present study was approved by the Ethical Committee on Animal Experimentation of Fundação Oswaldo Cruz (CEUA/FIOCRUZ) under the license No. LW-11/10 (protocol No. P-28/10-3). All the procedures followed the technical rules established by the Federal Board of Veterinary Medicine (CFMV resolution No. 714/2002). The dog owners were previously informed of the project objectives and signed a Statement of Informed Consent regarding sample collection for biopsy.

2.2. Study area

Sabará (19°53′21″ S 43° 48′ 17″ W) is part of the Metropolitan region of the state capital Belo Horizonte (Fig. 1). The town occupies an area of 302 km^2 with 64 districts and about 134.300 inhabitants. Eight districts of Sabará—named Alvorada, Novo Alvorada, Alvorada Velho, Bom Retiro, Rio Negro, Ana Lúcia, Casa Branca, and Nova Vista— were selected for study based on recent reports of canine and human cases of VL.

2.3. Canine census survey

To determine the current positivity rates of canine VL, two census population surveys were performed in 2011 and 2012. The blood samples were taken to the Zoonoses Laboratory of the Health Department of Sabará. The presence of *Leishmania* antibodies was investigated by enzyme-linked immunosorbent assay (ELISA) followed by indirect immunofluorescence assay (IFA), according to the standard protocol adopted by the Brazilian Ministry of Health at that time [9]. Only dogs with positive results in both tests were considered seropositive to VL. When the ELISA was positive but the IFA antibody titer remained in the borderline (1:40) the result was undetermined. The positivity rates were calculated by dividing the number of seropositive dogs in a given district by the number of tested dogs in the district.

The screening-and-culling procedure of the seropositive dogs was conducted by trained technicians from the Center for Zoonosis Control (CCZ) of Sabará and followed the rules established by the Brazilian Ministry of Health for VL control.

2.4. Euthanasia and tissue collection from seropositive dogs

Fifty seropositive dogs for VL from the canine census survey were randomly selected for further clinical examination by a veterinarian. The dogs were classified as asymptomatic or symptomatic, according to the absence or presence of, at least, one clinical sign of VL – cutaneous alterations such as alopecia, dermatitis and ulcers; onycogryphosis; keratoconjunctivitis; loss of weight; emaciation and rigidity of posterior limbs [10,11]. After anesthesia with Thionembutal (30 mg/mL *via* i.v.), bone marrow aspirates were harvested by sterilely puncturing the tibial crest. The aspirates were inoculated in the appropriate culture medium for parasite isolation and also used for the preparation of slide smears. Subsequently, the dogs received an intravenous injection of saturated solution of KCl (0,5 mL/kg). Biopsied samples from spleen, mesenteric lymph node and skin tissues were submitted to further parasitological and molecular analysis.

2.5. Leishmania isolation

Bone marrow aspirates (two samples per dog) were inoculated into Novy-MacNeal-Nicolle-liver infusion tryptose (NNN/LIT) culture medium and incubated at 25 °C. The cultures were weekly examined for the presence of *Leishmania* promastigotes. The positive samples were gradually expanded to 100 million cells. After washing with PBS, the *Leishmania* biomass was frozen at −20 °C until use. Negative samples were discarded after five weeks of monitoring. *Leishmania* growing in any of the two samples per dog was considered as a positive result for that dog.

2.6. Direct parasitological test

The presence of *Leishmania* amastigotes was microscopically investigated in bone marrow smears, and tissue—spleen, lymph node and skin—imprints, in this order, after slide staining by Giemsa. In case of *Leishmania* visualization in any of the samples, the result was taken as positive in the direct parasitological test.

2.7. Molecular analysis

Total genomic DNA was extracted from tissue—spleen, lymph node and skin—fragments and bone marrow aspirates using the

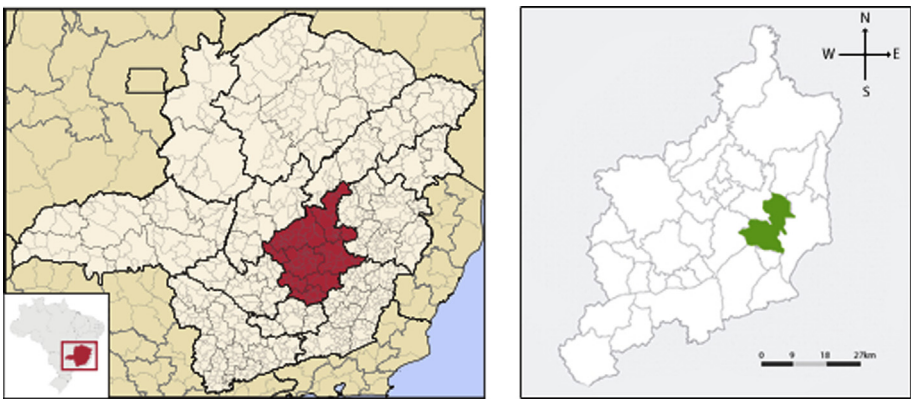

Fig. 1. Geographical localization of the municipality of Sabará, in the Metropolitan area of the state capital, in Minas Gerais, Brazil. The Metropolitan area of Belo Horizonte is marked in red (left map). Sabará is marked in green in the expanded map of the Metropolitan area (right map).