Epidemiology of canine leishmaniasis in southern Bahia, Brazil

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ABSTRACT

Leishmaniosis is a zoonosis caused by protozoa of the genus Leishmania. American cutaneous leishmaniosis (ACL) is mainly caused by the species L. amazonensis and L. braziliensis, and American visceral leishmaniosis (AVL) is caused by L. infantum chagasi. In addition to their proven roles as reservoirs of AVL, dogs are also suspected by researchers to be reservoirs of ACL due to reports of this infection in domestic environments and of infected dogs in endemic areas. The aim of this study was to detect Leishmania sp. infection in dogs from Vila Operária, Buerarema, Bahia, using parasitological tests, indirect immunofluorescent assay (IFA) and polymerase chain reaction (PCR). Furthermore, this study also aimed to identify risk factors associated with illness in dogs in this locality by conducting an epidemiological survey. For this purpose, 292 dogs were clinically evaluated for the presence of skin lesions, and the dogs that showed these changes were submitted to scarification injury to enable preparation of slides for microscopic study of amastigotes. Subsequently, the dogs underwent blood sampling for serological (IFA) and molecular (PCR) tests. Additionally, the owners of the dogs answered an epidemiological questionnaire to facilitate the identification of risk factors for exposure of dogs to pathogens of ACL. Of the 292 dogs studied, 13 (4.5%) had lesions suggestive of ACL, but with a negative parasitological examination and 147 (50.3%) were seropositive according to the IFA. Of the 273 dogs studied using PCR test, 10 (3.66%) were positive for L. braziliensis, and all samples were negative for L. infantum chagasi. Wastelands in the peridomicile and the presence of light in the household were risk factors associated with ACL. The results show that Vila Operária has asymptomatic dogs with ACL and that the detection sensitivity of the IFA was higher than that of PCR for the infected dogs.

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1. Introduction

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The leishmaniases are zoonoses caused by digenetic protozoa of the genus Leishmania (Dawit et al., 2013), that are divided into two categories: American visceral leishmaniosis (AVL) and American cutaneous leishmaniosis (ACL) (Ashford, 2000; Brasil, 2010; Chappuis et al., 2007; Heusser Júnior et al., 2010).

The cutaneous form of the disease is described from the southern tip of the United States to northern Argentina, except for Chile and Uruguay. In Brazil, seven disease causing species are recognized, with the most important being Leishmania (Vianna) braziliensis and Leishmania (Leishmania) amazonensis (Brasil, 2010). Data published by the Secretaria de Vigilância em Saúde (2011) **Q5** revealed that the North and Northeast regions were responsible for the vast majority of diagnoses recorded between the years 2000 and 2010, highlighting the state of Bahia, which recorded 10.2% of the human cases diagnosed in the country during this period. The high number of human cases of ACL that were associated with the diagnosis of this infection in dogs led some authors to suspect that such animals may be acting as a natural source of infection (Dantas-Torres, 2007; Falqueto et al., 1986; Heusser Júnior et al., 2010; Pittner et al., 2009).

The AVL, caused by *L. infantum chagasi*, in turn, is a disease of chronic evolution and, in Latin America, has been reported in at least 12 countries, with 90% of the cases of the disease on the continent concentrated in Brazil (Brasil, 2013; Chappuis et al., 2007; Maia-Elkhoury et al., 2008; Melo, 2004; Rondon et al., 2008; Tolezano et al., 2007). Infected dogs are considered reservoirs of the disease in rural and urban areas, having great ability to transmit the agent to the vector (Rodrigues et al., 2007).

Thus, considering the role of dogs as reservoirs of AVL and the possibility to also act as a reservoir of ACL, thereby influencing the transmission and maintenance of the disease, this study aimed to detect the presence of *Leishmania* sp. in dogs by parasitological, serological and molecular tests, as well as to identify factors associated with infection in an endemic area for human ACL in northeastern Brazil.

2. Materials and methods

2.1. Local search

The study was conducted between the months of March and August 2010 in the District of Vila Operária, City of Buerarema (Latitude: 14°56′ South, Longitude: 39°18′ west), southern Bahia, which is considered an endemic area for human ACL (Castellano et al., 2009). The Vila Operária is a rural district, approximately 7 km away from the town center of Buerarema, consisting of a human settlement of predominantly rural character, popularly known by residents as "Sururu," and other areas composed of farms. Vila Operária has a humid tropical climate, an average annual temperature of 26 °C and annual rainfall ranging from 1500 mm to 2000 mm. This locality is within the Atlantic forest, and the main livelihood is cocoa farming.

2.2. Animals

All canine populations residing in the locality were included in this study, excluding dogs less than six months of age, for a total of 292 dogs. After authorization of the owners, the dogs were initially assessed clinically for the presence of skin lesions, and subsequently, blood samples (approximately 5 ml) were collected by venipuncture of the cephalic or jugular veins. The samples were separated into two tubes without anticoagulant for conducting serological tests and into one tube with anticoagulant (EDTA), for molecular biology procedures. The methodology used in this research was judged and approved by the Comitê de Ética no Uso de Animais (CEUA) from the Universidade Estadual de Santa Cruz—UESC, under protocol no. 002/10.

2.3. Epidemiological data

A semistructured interview was conducted with the owners of the dogs with the aim of identifying factors associated with infection and covered issues such as review of the dog, characteristics of the environment in which the dog lives and contact with other animals (other dogs, cats, donkeys and rodents) (Almeida et al., 2009).

2.4. Parasitological exam

Dogs that presented with skin changes consistent with ACL during the clinical examination underwent scarification of lesions with the aid of scalpel blades. The collected material was distributed to glass slides, which were stained with fast Panoptic for microscopic observation of *Leishmania* sp. amastigotes (Dantas-Torres, 2006).

2.5. Serology

The tubes without EDTA were centrifuged at 1292 × g for 15 min to obtain serum. The serological technique used was immunofluorescence assay (IFA), and a kit for the diagnosis of Canine Leishmaniasis-Bio-Manguinhos® (Ribeiro et al., 2007) was used, following the manufacturer's recommended protocol. Titration of the conjugate was performed according to the guidelines of FUNED-Fundação Ezequiel Dias. Serologic titers ≥1:40 were considered positive.

2.6. Molecular biology

The blood samples with anticoagulant were centrifuged at 1292 × g for 15 min to separate the buffy coat. DNA was extracted from the buffy coat using the phenol-chloroform method and stored at −20 °C. After extraction, the DNA of all samples was measured using a spectrophotometer.

PCR was performed to detect the DNA of the etiological agent involved in the infection because there are reports in the literature of possible cross-reactivity in serological tests among agents of dermotropic leishmaniosis, visceral leishmaniosis and Chagas disease (Camargo and Rebonato, 1969; Luciano et al., 2009). For this, specific primers were used for the detection of DNA from *L. braziliensis* and *L. infantum chagasi*.

For detection of DNA from *L. braziliensis*, the sequences of the primers used were B1 (5′GGGGTTGGTGTAATATAGTGG 3′) and B2 (5′CTAATTGTGCACGGGGAGG 3′), which amplify a fragment of 750 bp of DNA from *Leishmania* (*Viana*) *braziliensis* (Bruijn and Barker, 1992). The conditions for the PCR reaction, including the total number of cycles; the denaturation, annealing and extension temperatures; and the concentrations of the MgCl2 and Taq DNA polymerase, were adapted from those previously described by Reithinger et al. (2000). The reaction was initiated in a final volume of 25 μl, composed of 17 μl Supermix® (Invitrogen), 1 U Taq DNA polymerase (Invitrogen), 0.6 mM MgCl (Invitrogen), 20 pmol of each primer and 100 ng of DNA. DNA amplification was performed using a thermocycler (*Biocycler* MJ96G) programmed for initial denaturation for 5 min at 94 °C; followed by 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 7 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photodocumented. A pure culture strain of *L. braziliensis* (MHOM/BR/3456) was used as a positive control and ultrapure water as a negative control.

For detection of DNA from *Leishmania infantum chagasi*, the sequences of the primers used were RV1 (5′CTTTTCTGGT-CCCGCGGGTAGG 3′), and RV2 (5′CCACCTGGCCTATTTTACACCA 3′), which amplify a fragment of 145 bp of DNA from *L. infantum chagasi* (Ravel et al., 1995). The conditions for the PCR reaction, such as the total number of cycles; the denaturation, annealing and extension temperatures; and the concentrations of the MgCl2 and Taq DNA polymerase, were adapted from those previously described by Lachaud et al. (2002). The reaction was conducted in a final volume of 25 μl, which was composed of 17 μl Supermix® (Invitrogen) 20 pmole of each primer and 100 ng of DNA. DNA amplification was performed according to the program used by Reithinger et al. (2000) with adaptations, with an initial denaturation of 5 min at 94 °C; followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 45 s; and a final extension of 72 °C for 7 min. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and photodocumented. A pure culture strain of *L. chagasi* (MHOM/BR2000/Merivaldo) was used as a positive control and ultrapure water as the negative control.