Leishmania sp. infection in dogs from Florianópolis, Santa Catarina, SC, Brazil

Infecção por Leishmania *sp. em cães de Florianópolis, Santa Catarina, SC, Brasil*

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Abstract

The aim of the present study was to investigate the occurrence of *Leishmania* sp. infection in dogs (N = 491) living in the municipality of Florianópolis, Santa Catarina (SC), Brazil, which was considered a disease-free region for visceral leishmaniasis until 2011, when autochthonous cases of canine disease were notified. Seroprevalence in this population was assessed by ELISA (0.4%; 2/491) and IFAT (4.09%; 24/491). Only one dog exhibited seroreactivity in both serological methods, comprising a total of 25 (5.3%) seroreagent animals. *Leishmania* sp. DNA, obtained from a sample of whole blood of this animal, was amplified by both conventional and Real-Time PCR. Sequencing of the amplified DNA and, thereby, determination of the *Leishmania* species involved, was not possible. Our results suggest the necessity of a thorough epidemiological investigation in Florianópolis.

Keywords: Visceral Leishmaniasis. Diagnosis. ELISA. IFAT. PCR.

Resumo

O objetivo do presente estudo foi pesquisar a ocorrência de infecção por *Leishmania* sp. em cães (N = 491) domiciliados no município de Florianópolis, Santa Catarina, considerada uma região indene para leishmaniose visceral até o ano de 2011, quando foram notificados casos autóctones da doença canina. A soroprevalência na população foi avaliada por ELISA (0,4%; 2/491) e RIFI (4,09%; 24/491). Somente um cão apresentou sororeatividade em ambos os métodos sorológicos, totalizando 25 (5,3%) animais sororeagentes. O DNA de *Leishmania* sp., obtido de uma amostra do sangue total desse animal, foi amplificado por PCR convencional e PCR em Tempo Real. Não foi possível realizar o sequenciamento do DNA amplificado e, deste modo, determinar a espécie de *Leishmania* envolvida. Os nossos resultados sugerem a necessidade de uma investigação epidemiológica minuciosa em Florianópolis.

Palavras-chave: Leishmaniose Visceral. Diagnóstico. ELISA. RIFI. PCR.

Introduction

Since 1913, when a case of American visceral leishmaniasis (AVL) was first described in a patient in the state of Mato Grosso, Brazil, new cases of AVL have been reported in regions where the disease did not occur. Currently, AVL affects almost all states of Brazil (BRASIL, 2006a,b). While in 1994, 92.9% of the cases were concentrated in the northeast and only 2.6% in the southeast of Brazil, with the territorial expansion of the disease, in 2006 the distribution of human cases was 56.2% in the northeast and 21% in the southeast regions (BRASIL, 2006a). In addition, AVL, which had a rather rural distribution, was also identified in several Brazilian urban cities, such as Corumbá (MS), Belo Horizonte (MG), Araçatuba (SP), Palmas (TO), and Três Lagoas (MS) (BRASIL, 2006a). In part, this is due to a high adaptive capacity of *Lutzomyia longipalpis*, the main vector incriminated in the transmission of this disease in Brazil (BRASIL, 2006a).

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Although *Lutzomyia longipalpis* is usually related to transmission of visceral leishmaniasis (VL) in several Brazilian states, infections by *Leishmania infantum chagasi* in other phlebotominae, such as *Lutzomyia cruzi* and *Nyssomyia neivai*, have been reported. These infections may also suggest involvement of these phlebotominae in the disease transmission (BRASIL, 2010b; BRASIL, 2006a; SARAIVA et al., 2009).

Dogs are the main domestic reservoir of VL, being responsible for maintaining the agent in endemic regions. Usually, canine cases precede the appearance of human cases, which incidence ranges from 1 to 2% in endemic areas (SOLANO-GALLEGO et al., 2009). Dogs are important in the epidemiology of the disease not only because their infections have a higher prevalence when compared to humans, with rates that can reach 40% of the population, but also due to the high number of asymptomatic animals, which can reach 80% of the infected dogs (BANETH, 2008; PALTRINIERI, 2010). These act as sources of infection for the vector, and are often not identified in the population due to the absence of clinical signs or to false-negative results in serological tests (BANETH et al., 2008).

The southern region of Brazil was considered free from visceral leishmaniasis until recently, when there were only reports of the disease in its cutaneous form, in both humans and animals (HEUSSER-JÚNIOR et al., 2010; ROSSETO et al., 2006). In 2007, two municipalities (Cruz Alta and Uruguaiana) in the state of Rio Grande do Sul (RS) had alochthonous cases of the disease (visceral form) in dogs (DALMOLIN et al., 2008; KRAUSPENHAR et al., 2007). Two years later, an outbreak of the disease occurred in the municipality of San Borja (RS) with over 1200 seropositive dogs (TARTAROTTI et al., 2011) and a notification of five autochthonous cases in humans (BRASIL, 2010a).

In the state of Santa Catarina (SC), the first cases of cutaneous leishmaniasis in humans were reported in 1990. Since then, an expansion of the disease occurred, with autochthonous cases described in humans and dogs in the municipalities of Balneário Camboriú, Chapecó, and Blumenau (SC), with no reported cases in Florianópolis, capital of SC (HEUSSER-JÚNIOR et al., 2010; LIMA-FILHO; STEINDEL, 1998; ROSSETO et al., 2006). Nevertheless, in 2010, Correa et al. evaluating 102 dogs by serological methods identified *Leishmania* sp. antibodies in seven of them, all from the same neighborhood, Lagoa da Conceição (CORRÊA et al., 2011). However, confirmation of the species of *Leishmania* involved in these cases was not possible. Two years later, Figueiredo et al. (2012), using immunoenzymatic methods, confirmed infection by *Leishmania infantum chagasi* in two of the seven dogs identified by serology in 2010 (FIGUEIREDO et al., 2012).

Regarding the geographical dispersion of visceral leishmaniasis and case reports of the disease in dogs, the aim of this study was to investigate, by serology and polymerase chain reaction, the occurrence of *Leishmania infantum chagasi* infection in a population of dogs from Florianópolis (SC).

Materials and Methods

The present study was approved by the Committee for Ethics in Animal Research (São Paulo State University, Araçatuba, SP, Protocol Nº 01248). For convenience of the investigators, samples of whole blood and serum from dogs (N = 491) older than six months, referred for elective surgical procedures to the Center for Zoonosis Control (CZC) of Florianópolis (SC), were selected, regardless of their gender or breed, during 2011. After informed consent of the owners, a blood sample was collected from each animal for detection of both *Leishmania infantum chagasi* DNA and serum *Leishmania* antibodies. Only dogs born in Florianópolis, SC, which never left the city and were not previously vaccinated against VL were included in the study. All animals referred for surgical procedure were previously examined by veterinarians, and were considered healthy. Animals that showed any alteration on physical examination

were referred for a complete clinical evaluation in another sector.

Serum antibodies were detected by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent antibodie test (IFAT). The ELISA was carried out as described by Lima et al. (2003), using *L. infantum chagasi* total promastigote lysate (strain MHOM/BR/74/PP75) and peroxidase-labeled goat anti-dog IgG antibody (A40-123P, Bethyl, Montgomery, USA) diluted 1:80,000 in PBS-Tween. All samples were assayed in duplicate and the results were expressed by the mean of values for optical density read in two reactions. The cutoff point (0.274) of the reaction was determined by evaluating samples of serum from 30 dogs residing in an area non-endemic for VL (mean+3 SD).

IFAT was performed as described by Silva et al. (2009), by using a suspension of *Leishmania infantum chagasi* promastigotes and fluoresceinated anti-dog IgG antibody (FITC conjugate; Sigma Aldrich, Brazil) diluted 1:100 in PBS containing Evan's Blue. Samples with dilution equal to or greater than 1:40, which showed fluorescence reaction, were considered positive.

In animals with positive sera, peripheral blood was investigated for the presence of *Leishmania* sp. DNA by means of real-time polymerase chain reaction (qPCR), as described in the literature (VIDES et al., 2011).

Serum samples that reacted in either ELISA or IFAT were examined for the detection of *Dirofilaria immitis* antigens and *Ehrlichia canis* antibodies by using a commercial kit (SNAP 4DX® test, IDEXX Laboratories Inc., Westbrook, ME, USA), and were also evaluated using an immunochromatographic qualitative antibody assay against *L. chagasi* recombinant K39 (rK39) antigen (Kalazar Detect®, InBios Inc., Seattle, WA, USA) according to the manufacturer's protocol.

Results and Discussion

A total of 491 dog sera samples, from 52 (61%) of the 85 neighbourhoods of the municipality, were evaluated. Among the evaluated samples, sera from 5.3% (25/491) animals were considered positive by ELISA (0.4%; 2/491) and IFAT (4.09%; 24/491). Regarding both tests, the values for seroprevalence are close to those reported in previous studies (CORRÊA et al., 2011), in which a seroreactivity of 6.86% (7/102) was observed in dogs from the same municipality. Although the results of both studies are similar, the first was conducted in a single neighborhood (Lagoa da Conceição) of the municipality where Figueiredo et al. (2012) confirmed that *Leishmania infantum chagasi* was the etiologic agent in dogs previously identified. Although the occurrence of the disease has been previously identified in this neighborhood, seroreactivity was not observed in any of the samples obtained in the present study. However, as the animals were from the CZC, the number of samples from that neighborhood resulted smaller than that used in the previous evaluation, comprising only five animals.

Seropositive dogs were identified in 13 neighbourhoods, which belong to seven different administrative districts, suggesting a diffusion of the disease within the municipality. Among the evaluated dogs, 27% (136/491) were from the Sede Insular district, where the presence of *Leishmania* sp. DNA was detected in a dog residing in the neighborhood of Santa Monica, which has a high population density. In the remaining districts, although the occurrence of dogs with serum anti-*Leishmania* sp. antibodies was observed, no parasite DNA was amplified in blood samples.

As these dogs live in a seaside town where there are cases of canine dirofilariasis and there is a report of serological cross-reaction between anti-*Leishmania* sp. and anti-*E. canis* antibodies while using the ELISA technique (ZANETTE et al., 2008b), the possibility of cross-reactivity between these agents was evaluated. However, this possibility was ruled out because *D. immitis* antigens and anti-*E. canis* antibodies were not detected in the seropositive animals. Occurrence of cross-reaction involving causative agents of canine babesiosis was also ruled out because cross-reaction involving anti-*Leishmania* sp. antibodies with the serological methods used in this study were not found

in the literature (ZANETTE et al., 2008c). Similarly, the possibility of cross-reaction with *Trypanosoma cruzi*, as described in a previous study (VIOL et al., 2012), was discarded because the dogs evaluated in that study had never left the city of Florianópolis, where neither the vector was identified nor a notification of cases of Chagas disease exists until the present date. Furthermore, all animals were also considered positive when evaluated by the Kalazar Detect® test, which showed no cross-reaction between the anti-*Trypanosoma cruzi* and anti-*Leishmania* sp. antibodies, as observed in a previous study (ZANETTE et al., 2008b). However, the possibility of cross-reaction with antibodies against etiological agents of cutaneous leishmaniasis, such as *L. braziliensis* and *L. amazonensis*, cannot be ruled out, because there are reports of indigenous cases of the disease in both humans and dogs living in the state of Santa Catarina (GRISARD et al., 2000; HEUSSER-JÚNIOR et al., 2010).

Despite real-time PCR sensitivity, used in the present study, is higher than that of conventional PCR, its sensitivity is usually increased when investigation is performed in samples from organs such as lymph nodes, bone marrow, spleen, and liver (PALTRINIERI et al., 2010; QUEIRÓZ et al., 2010); however, samples of those organs were not collected. Sequencing of the amplification products could not be carried out in the only blood sample in which the parasite DNA was amplified, since the amount of extracted DNA was insufficient. It was also not possible to obtain samples from this dog lymphoid organs, since it had already been euthanized due to a positive serological result in the test officially adopted by the Brazilian Ministry of Health. Therefore, it is not possible to state that the parasite was *Leishmania infantum chagasi*.

It is worth emphasizing that all dogs evaluated in this study were healthy, which decreases the probability of seroconversion in case of infection by *L. infantum chagasi*. Infected animals, in which the cellular response is predominant, do not develop symptoms of the disease and not always have serum antibodies (BANETH et al., 2008; PALTRINIERI et al., 2010). Thus, it is possible that occurrence of infection in the municipality has been underestimated, since symptomatic dogs were not included in the evaluation. Moreover, as the humoral immune response can occur within about two years after infection (PALTRINIERI et al., 2010; SOLANO-GALLEGO et al., 2005), and if VL is starting its expansion in that municipality infected dogs may not yet have serum antibodies.

Conclusion

Results of this study confirm the occurrence of autochthonous cases of leishmaniasis in dogs from Florianópolis, SC. Our results also suggest that thorough epidemiological investigation and intensification of preventive measures should be done in an attempt to prevent spread of the disease in the municipality.

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