Veterinary Parasitology 164 (2009) 118–123

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Leishmania spp. and/or *Trypanosoma cruzi* diagnosis in dogs from endemic and nonendemic areas for canine visceral leishmaniasis

Marcella Zampoli Troncarelli [a,*], Janaína Biotto Camargo [a], Juliana Giantomassi Machado [a], Simone Baldini Lucheis [b], Helio Langoni [a]

[a] *School of Veterinary Medicine and Animal Science – FMVZ, Sao Paulo State University – UNESP, Distrito de Rubião Júnior, s/n, Botucatu, São Paulo 18618-000, C.P. 560, Brazil*

[b] *Agência Paulista de Tecnologia dos Agronegócios – APTA, Pólo Centro Oeste, Av. Rodrigues Alves, 40-40, 17030-000 Bauru, São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 31 July 2008
Received in revised form 30 May 2009
Accepted 13 June 2009

Keywords:
Leishmania spp.
Trypanosoma cruzi
Diagnosis
Dog

ABSTRACT

Due to the phylogenetic similarity between *Leishmania* spp. and *Trypanosoma cruzi* (*T. cruzi*), serological cross-reactions and false-positive results are quite common. This study aimed to elucidate canine leishmaniasis and trypanosomiasis diagnosis by the indirect fluorescent antibody test (IFAT) on serum samples, and direct parasitological examination and polymerase chain reaction (PCR) in liver and spleen samples. One hundred dogs from Zoonosis Control Center (ZCC) in Bauru, SP, an endemic area for visceral leishmaniasis (VL), and 100 dogs from the Dog Warden Service in Botucatu, SP, a nonendemic area for VL, were studied. IFAT showed positive results for *Leishmania* spp. in 65% of canine serum samples from Bauru while 40% of the samples were positive for *T. cruzi* by this test. All samples from Botucatu were negative for leishmaniasis in IFAT, and only 4% were positive for *T. cruzi*. Out of 200 serum samples tested, 33 (16.5%) showed positive serological results for both the parasites. Direct parasitological examination and PCR found, respectively, 59% and 76% of the liver samples and 51% and 72% of the spleen samples of dogs from Bauru positive for *Leishmania* spp. Twenty-six (78.8%) of 33 dogs that showed anti-*Leishmania* spp. and anti-*T. cruzi* antibodies also tested positive by direct parasitological examination and PCR for *Leishmania* spp., which indicates that these dogs presented leishmaniasis. No liver or spleen sample from the 200 dogs analyzed showed a positive PCR result for *T. cruzi*. These findings support the occurrence of cross-reactions between *Leishmania* spp. and *T. cruzi* in IFAT; they also corroborate the need for simultaneous PCR and/or parasitological examination to establish canine leishmaniasis (CL) diagnosis.

1. Introduction

Dogs are the main domestic reservoirs of *Leishmania* spp. due to their intense skin parasite load, their close contact with humans and their ability to attract the vector (Machado, 2004). One of the control measures recommended by the Brazilian Ministry of Health is the culling of dogs found positive in serological surveys, especially in endemic areas (Brasil, 2006). The indirect fluorescent antibody test (IFAT) is officially recommended for canine diagnosis confirmation, showing sensitivity and specificity values ranging from 90% to 100% and 80% to 100%, respectively (Alves and Bevilacqua, 2004). This test is feasible, fast and inexpensively executed (Ikeda-Garcia and Feitosa, 2006).

Other techniques, therefore, such as direct parasitological examination and polymerase chain reaction (PCR), which detects the parasite's DNA, are necessary to establish the diagnosis. Sensitivity of direct parasitological exam is directly related to parasite load, biological material

* Corresponding author. Tel.: +55 14 3811 6270x24; fax: +55 14 3811 6075.
E-mail addresses: matroncarelli@yahoo.com.br, matroncarelli@hotmail.com (M.Z. Troncarelli).

0304-4017/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2009.06.027

collected, technical experience and time for slide examination (Ikeda-Garcia and Feitosa, 2006). This test presents sensitivity values of 58%, 70% and 96%, for lymph node, bone marrow and spleen aspirates, respectively (Zijlstra et al., 2001). However, its specificity is 100% and for this reason the direct parasitological exam is considered to be the gold standard. On the other hand, PCR diagnosis does not depend on the dog's immunological status and can be used in cases of inconclusive reactions, anergy or cross-reactions in serological tests, and shows sensitivity and specificity values near 100% (Ashford et al., 1995; Lachaud et al., 2002).

Bauru, SP (Brazil) is an endemic area for canine leishmaniasis (CL). In 2007, a total of 4325 dogs were culled at the Zoonosis Control Center (ZCC), of which 3407 were leishmaniasis-seropositive. From January to March 2008, 651/768 dogs euthanized were serologically positive (São Paulo, 2006). However, there is a lack of studies about CL and Chagas prevalence in Bauru.

Although Botucatu, SP (Brazil) is a nonendemic area for CL (Langoni et al., 2001; São Paulo, 2006), Chagas disease in dogs has been detected there (Lucheis et al., 2005).

Due to the phylogenetic similarity between *Leishmania* spp. and *Trypanosoma cruzi*, serological cross-reactions and false-positive results are quite common (Troncarelli et al., 2008). Improvement of diagnosis methods for the correct identification of canine infection is necessary to better comprehend the disease's status in dogs and also to contribute to its control. Thus, the aim of the present study was to evaluate the occurrence of cross-reactions between *Leishmania* spp. and *T. cruzi* in serum samples collected from dogs found in Bauru and Botucatu, using the IFAT, and also to use the direct parasitological examination and PCR in liver and spleen samples as complementary methods for diagnosis elucidation.

2. Material and methods

2.1. Animals and samples

One hundred dogs of mixed breed (53 males and 47 females), culled at the Dog Warden Service in Botucatu, and 100 dogs of unidentified breed (50 males and 50 females), culled at ZCC in Bauru were used in the present study.

Serum samples were obtained from 5 to 10 mL of the blood collected from each animal and centrifuged at 3590 g for 10 min. Samples were kept at −20 °C until they were submitted to IFAT.

Liver and spleen fragments, weighing approximately 100 g, were collected immediately after the animals have been culled. At the Laboratory for Diagnosis of Zoonoses at the School of Veterinary Medicine and Animal Science (FMVZ), in the Sao Paulo State University (UNESP)/ Botucatu, SP, fragments of about 1 g were removed from each sample, and placed in 1.5 mL sterile, DNAse and RNAse-free microtubes containing 1 mL of sterile phosphate buffered saline (PBS) 0.01 M, pH 7.2 (Nicolas et al., 2002). These fragments were kept at −20 °C until the DNA extraction for PCR.

Samples were collected between April 2006 and February 2007 at ZCC in Bauru, and from January to April 2007 at the Dog Warden Service of Botucatu, SP.

2.2. Indirect fluorescent antibody test (IFAT)

The *L. major* antigen kept by the Zoonoses Research Nucleus (NUPEZO) at FMVZ-UNESP/Botucatu, SP, was used in the IFAT for anti-*Leishmania* spp. antibodies (Rosypal et al., 2007). The entire *Leishmania* organism (promastigote) was fixed on the slides. This antigen cross-reacts with all the *Leishmania* species circulating in Brazil (Barbosa-de-Deus et al., 2002). Serum samples, as well as positive and negative controls were diluted in PBS, pH 7.2, starting from 1:40. Samples were considered positive when titers were 40 or higher.

The *T. cruzi* Y strain, kept by NUPEZO was used for the assessment of anti-*T. cruzi* antibodies (Crisante et al., 2006). The entire *T. cruzi* organism (epimastigote) was fixed on a slide. Serum samples, as well as positive and negative controls were diluted in PBS, pH 7.2, starting from the 1:20. Samples were considered positive when titers were 20 or higher.

2.3. Direct parasitological examinations

Imprints of liver and spleen fragments were stained by Giemsa and observed in an optical microscope at a 1000× magnification. Three slides were imprinted with each fragment. Positive results were obtained when at least one complete parasite amastigote form (containing nucleus, cytoplasm and kinetoplast) was observed in liver and/or spleen fragments after careful examination of the slides. If no amastigote form was found in any of the three slides evaluated for each fragment, then the results were considered negative.

Direct parasitological examination was considered the gold standard for calculation of sensitivity and specificity values of all tests used.

2.4. Polymerase chain reaction (PCR)

2.4.1. DNA extraction

Liver and spleen fragments were individually macerated using sterile mortars and pestles, in a laminar flow bench. A 4-mL quantity of sterile PBS (0.01 M, pH 7.2) was added, and the solution was carefully homogenized. The supernatant was transferred to a sterile, DNAse and RNAse-free microtube, and kept at −20 °C until DNA extraction by means of the GenomicPrep Cells and Tissue DNA Isolation Kit® (Amersham Biosciences), according to the manufacturer's instruction.

2.4.2. PCR for the analysis of Leishmania spp. DNA

PCR procedures were carried out at the Laboratory of Applied Molecular Biology for Zoonosis Diagnosis (FMVZ-UNESP/Botucatu, SP). The PCR mixture was composed of 5 μL of 10× PCR buffer minus Mg/1 mL (50 mmol KCl, 10 mmol Tris–HCl), 8 μL dNTPs [25 mM (250 μL), 1:20], 1.5 μL magnesium chloride (50 mM/1 mL), 0.3 μL *Platinum Taq*-polymerase [500 U (5 U/μL)], 5 μL of LIN R4 primer (10 pmol; MW = 6317.1; 28.7 nmol = 0.18 mg) and 5 μL of LIN 19 primer (10 pmol; MW = 5085.3; 80.6 nmol = 0.41 mg), 15.2 μL ultrapure water and 10 μL of the test sample [DNA extraction product (100 μg/mL)]. The mixture