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Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples

Arleana B.P.F. Almeida [a,b], Valéria R.F. Sousa [b], Naiani D. Gasparetto [b], Givago F.R. da Silva [b], Fabiano B. Figueiredo [c], Valéria Dutra [b], Luciano Nakazato [b], Maria F. Madeira [d,*]

[a] Programa de pós-graduação em Pesquisa Clínica em Doenças Infecciosas, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, 21040–900 Rio de Janeiro, RJ, Brazil
[b] Departamento de Clínica Médica Veterinária, Faculdade de Agronomia, Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso, Av. Fernando Correa da Costa 2367, Boa Esperança, 78060–900 Cuiabá, MT, Brazil
[c] Laboratório de Pesquisa Clínica em Dermatozoonoses de Animais Domésticos, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, 21040–900 Rio de Janeiro, RJ, Brazil
[d] Laboratório de Vigilância em Leishmanioses, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, 21040–900 Rio de Janeiro, RJ, Brazil

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ABSTRACT

The accurate diagnosis of canine visceral leishmaniasis (CanL) is essential for visceral leishmaniasis control. To this end, DNA detection on different biological samples has been employed. In this study, we report the use of polymerase chain reaction (PCR) assay on samples such as buffy coat, bone marrow, intact skin and cutaneous ulcers fragments, and lymph node aspirate collected from 430 dogs to determine the suitable biological sample for use in CanL diagnosis. The PCR results were correlated with clinical status and other tests previously performed. *Leishmania chagasi* DNA was detected in 14.6% (n = 63) of the dogs investigated, regardless of the sample analyzed. Our results showed that symptomatic cases were easily diagnosed when compared to asymptomatic animals; however, the PCR proved to be very useful for *Leishmania* DNA detection, mainly in lymph node aspirate (41; 9.6%), irrespective of the clinical status of the dog. The finding that the lymph node aspirate produced high positivity rates and the fact that this specimen was obtained by noninvasive methods highlight its use in epidemiological survey by PCR for CanL diagnosis.

1. Introduction

Visceral leishmaniasis (VL) is a zoonosis with high prevalence in Latin American countries (WHO, 2012). The domestic dog is considered an important reservoir in the transmission cycle of VL, and regardless of the clinical manifestation, it is a source of infection for Phlebotominae vectors (Ministério da Saúde, 2006).

In Brazil, VL occurs in urban and periurban areas and requires compulsory notification. One of the control measures recommended is the euthanasia of dogs seroreactive to *Leishmania* spp. (Ministério da Saúde, 2006). Therefore, accurate tests with high sensitivity and specificity are essential for the diagnosis of this disease. Recently, a fast immunochromatographic test—Dual Path Platform—began to be used in Brazil as a screening test in routine dog diagnosis (Ministério da Saúde, 2011). However, the confirmation of the infection through parasite and/or DNA detection becomes important, mainly in areas of overlapping of different agents (Silva et al., 2011) or areas where the disease has been recently installed (Souza et al., 2009). Biological samples such as blood, aspirates, and tissue fragments from different organs can be used for parasitological research of *Leishmania* spp.; however, there is still no agreement on which should be the standard sample. Nevertheless, the simplicity of collecting it, with less invasiveness, and the laboratory method used must be considered (Saridomichelakis, 2009). We cannot fail to mention that aspects such as the sensitivity and specificity of the diagnostic test may vary as a function of the clinical sample analyzed and the clinical status of the dog (Martínez et al., 2011). In this context, polymerase chain reaction (PCR) appeared as an alternative because it represents a gain in diagnosis sensitivity and specificity, and it is very useful, mainly in cases not resolved by the classical methods (Gomes et al., 2007; Lachaud et al., 2002; Moreira et al., 2007).

In the present study, we aimed to evaluate PCR as a tool for *Leishmania chagasi* diagnosis using different biological samples collected from domestic dogs from canine visceral leishmaniasis (CanL) endemic areas.

* Corresponding author. Tel.: +55-21-3865-9541; fax: +55-21-3865-9541.
E-mail address: fatima.madeira@ipec.fiocruz.br (M.F. Madeira).

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2. Materials and methods

2.1. Animals and biological samples

Four hundred thirty animals were evaluated in a transversal study performed in the municipality of Cuiabá, state of Mato Grosso (MT), midwestern Brazil (Almeida et al., 2011). The dogs were clinically examined and classified as symptomatic, oligosymptomatic, and asymptomatic according to Mancianti et al. (1988).

After the signing of the informed consent form by the owners, the dogs were mechanically restrained and subjected to sedation with ketamine (10 mg/kg) associated with acepromazine (0.2 mg/kg). Blood, bone marrow, lymph nodes aspirates, and tissue fragments (intact skin and cutaneous ulcers) were collected from all the animals for PCR processing. Around 5 mL of blood were collected from each animal by cephalic or jugular venipuncture. The buffy coat was separated by centrifugation, and the bone marrow (0.5 mL) was obtained by aspiration from the manubrium of the sternum—both with anticoagulants. The ganglionar aspirates were obtained from popliteal lymph nodes with Valeri cytoaspirator and placed in microtubes containing 250 μL of sterile saline solution. The tissue biopsies were collected after local anesthesia with 2% lidocaine, and the fragments were also placed in sterile microtubes. All biological samples were kept at −20 °C until use.

All the procedures in this study were approved by the Ethics Committee on Animal Use of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ/52/2009-3, protocol LW-01/10).

2.2. DNA extraction and PCR

DNA extraction was performed by the phenol/chloroform/isoamyl alcohol method according to Gomes et al. (2007). Briefly, the samples collected by puncture (buffy coat, bone marrow, and lymph node) and the tissue fragments (intact skin and cutaneous ulcers) were dissolved in lysis buffer containing 10 mmol/L Tris–HCl, pH 8.0; 10 mmol/L EDTA; 0.5% SDS; 0.01% *N*-laurilsarcosinate, and 100 μg/mL of proteinase K. Then, they were strongly shaken in vortex and incubated at 56 °C for 12–18 hours. After washing with 70% ethanol for 10 minutes at 10000 × g, the DNA precipitate was placed in ultra-pure water containing 20 μg/mL of RNAse and stored at −20 °C until use in PCR assays.

Initially, we used the 150 (sense) primers 5'-GGG(G/T) AGGGGCGTTCT(C/G)CGAA-3' and 152 (antisense) 5' (C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTACACCAACCCC-3' that amplify a product of 120pb of variable regions of kDNA minicircles of the *Leishmania* genus (Degrave et al., 1994). For the reaction, 200 μmol/L of dNTP, 1 μmol/L of each primer (10 mmol/L Tris–HCl, 50 mmol/L KCl, pH 8.3) buffer solution, 2.5 mmol/L $MgCl_2$, 2.5 U of Taq DNA polymerase, and 2 μL of DNA were used in a final 20 μL volume. The PCR conditions were as follows: initial denaturation at 94 °C for 4 minutes, followed by 30 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes.

A second PCR assay was carried out with the samples where amplification occurred in order to confirm the presence of *L. chagasi* DNA. At this time, the primers RV1- 5'-CTT TTC TGG TCC CGC GGG TAG G-3' and RV2 - 5'-CCA CCT GGC TAT TTT ACA CCA-3' that amplify a sequence of 145pb, specific of *L. chagasi*, were used (Lachaud et al., 2002). The PCR conditions were as follows: initial denaturation at 94 °C for 4 minutes, followed by 30 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes. In all PCR assays, DNA of reference strains of *Leishmania braziliensis* (MHOM/BR/75/M2903), *Leishmania amazonensis* (IFLA/BR/67/PH8), and *L. chagasi* (MHOM/BR/1974/PP75) were used.

The amplified products were fractionated by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized in a transluminator (UV-300nm).

2.3. Result analysis

The results obtained were transferred to a database and statistically analyzed by the EpiInfo 3.3.2 (CDC, Atlanta, GA, USA) software program. Differences in the frequencies of positive results for each clinical sample, comparative analyses of PCR results among different clinical samples, and comparisons with clinical status of dogs were performed using the chi-square test. In order to evaluate the index of agreement between the PCR and parasitological culture (Almeida et al., 2011), Kappa measurement (k) was used according to the classification proposed by Shrout (1998).

3. Results

From the 430 dogs evaluated, samples of buffy coat and intact skin fragments were collected from 100% of the animals, while bone marrow and lymph node aspirates were obtained from 429 (99.8%) and 427 (99.3%) animals, respectively. Twenty-two (5.1%) dogs presented cutaneous ulcers, located in the ear (14), back limbs (3), scrotum (2), nostrils (2), and upper lip (1).

Clinically, 42 (9.8%) dogs were symptomatic, presenting clinical signs such as severe weight loss, generalized lymphadenomegaly, splenomegaly, onychogryphosis, and dermatologic and ophthalmic disorders, while 150 (34.9%) were oligosymptomatic, with weight loss, lymphadenomegaly, and localized alopecia as the most frequent clinical signs, and 238 (55.3%) animals were considered asymptomatic.

Leishmania spp. DNA was detected in 63 (14.6%) dogs, regardless of the sample analyzed, and *L. chagasi* DNA was confirmed in all samples when a specific primer was used. From those animals, 13 (30.9%) were symptomatic; 23 (15.3%), oligosymptomatic; and 27 (11.3%), asymptomatic for CanL. In the Fig. 1, the pattern of amplification of both PCR assays can be observed.

The PCR was positive in 2 or more samples in 17 (27%) dogs, in 2 samples in 20 (31.7%) dogs, and in just 1 sample in 26 (41.3%) dogs. The lymph node (41; 9.6%) was the sample that presented the highest positivity rate, followed by intact skin (27; 6.3%), bone marrow (26; 6.1%), and buffy coat (20; 4.6%). *Leishmania* DNA was detected in 13 (59.1%) out of the 22 cutaneous ulcers fragments collected from the dogs (Table 1).

PCR proved to be statistically higher for *Leishmania* DNA detection in symptomatic animals compared to oligosymptomatic ($P < 0.001$) and asymptomatic ($P < 0.001$) ones, with likelihood 4.85 (2.17–11.03) and 10.43 (4.7–23.53) times greater, respectively. The likelihood of *Leishmania* spp. DNA detection in oligosymptomatic dogs was 2.15 (1.31–3.52) times greater than in asymptomatic ($P = 0.001$) animals. However, when each sample was evaluated separately, intact skin, bone marrow, and lymph node samples were statistically better for the detection of the agent in symptomatic animals when compared to asymptomatic ones ($P = 0.02$, 0.01, and 0.007, respectively). Although buffy coat was the sample with the largest number of positive results in asymptomatic dogs, it was not statistically reliable in others animals ($P > 0.05$).

When we compared the PCR results in different clinical samples, we observed that there was no statistical difference between asymptomatic and symptomatic dogs. However, the lymph node sample was statistically higher for *Leishmania* DNA detection compared to the buffy coat sample ($P = 0.04$; OR 2.91 [1.03–8.6]) in oligosymptomatic dogs.

From the 430 dogs evaluated, *L. chagasi* was isolated in 12 animals in parasitological culture (Almeida et al., 2011), and they were all positive in PCR. Such tests presented low agreement (k 0.287 [0.155–0.418]).

4. Discussion

The importance of dogs in the VL transmission cycle is widely recognized (Marzochi et al., 2009; Ministério da Saúde, 2006),

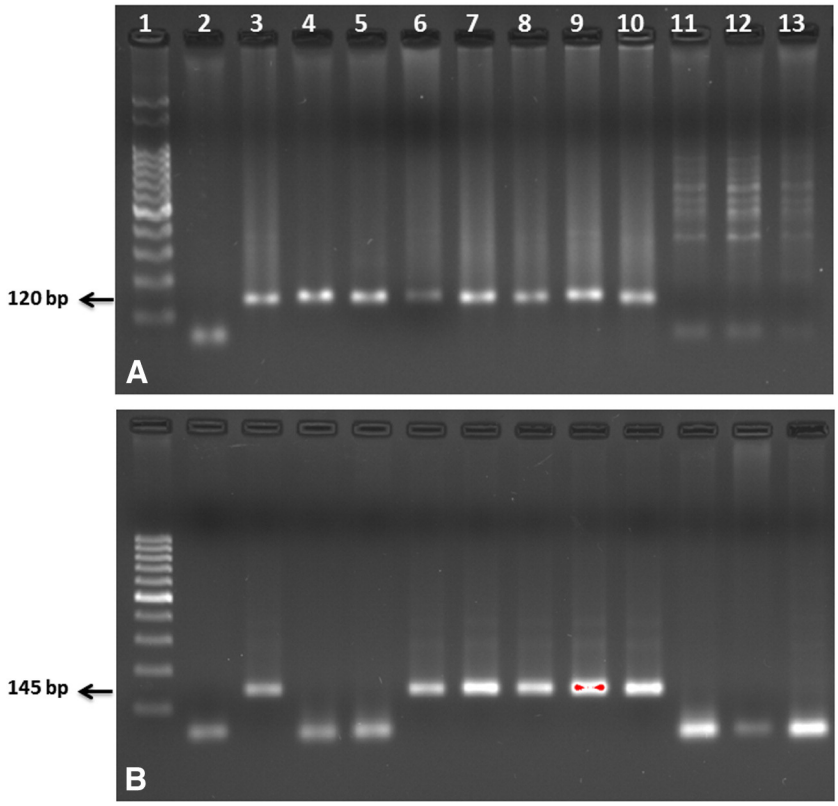

Fig. 1. Analysis of DNA amplification of PCR assays in 2% agarose gels employing generic primers for *Leishmania* genus (A) and specific primers for *L. chagasi* (B). Line 1 = DNA Ladder 100 bp; line 2 = negative control; line 3 = *L. chagasi* reference strain; line 4 = *L. braziliensis* reference strain; line 5 = *L. amazonensis* reference strain; lines 6–10 = positive samples: intact skin, cutaneous lesion, bone marrow, lymph node aspirate, and buffy coat; lines 11–13 = negative samples: intact skin, buffy coat, and lymph node aspirate.

concentrating efforts in research diagnostic methods that can discriminate infected dogs (Coura-Vital et al., 2011; Fisa et al., 2001; Gomes et al., 2007; Grimaldi et al., 2012; Moreira et al., 2007; Teles et al., 2012). In the present study, we confirmed *Leishmania* infection by PCR in 14.6% (n = 63) of dogs in the municipality of Cuiabá, where *L. chagasi* had been previously isolated and identified (Almeida et al., 2011).

Although little used in epidemiological surveys, molecular techniques have shown higher prevalence than serological methods (Coura-Vital et al., 2011). According to Oliva et al. (2006), this can be related to the sensitivity of PCR in detecting infection even before the occurrence of seroconversion. In Cuiabá, the serological prevalence for CanL has varied from 3.4 to 22.1%, depending on the area investigated (Almeida et al., 2009; 2012). One of the disadvantages related to the use of only serological tests for diagnosis of *Leishmania* infection in dogs is the possibility of cross-reactions, mainly in areas of overlapping with other trypanosomatids (Alves et al., 2012; Vexenat et al., 1996). In this context, *Trypanosoma caninum*, a species recently described in Rio de Janeiro municipality (Madeira et al., 2009b), was found in 14 out of the 430 dogs studied (Almeida et al., 2011; Barros et al., 2012), reinforcing the importance of using more accurate diagnostic methods.

Table 1
Parasitological culture and PCR results of the investigation of *L. chagasi* DNA, in different biological samples obtained from 430 dogs.

Clinical status	Positive culture[a]	PCR					
		BC	LN	BM	Skin	Ulcers	Total +/n
Asymptomatic (n = 238)	04	11	15	09	10	01	27/238
Oligosymptomatic (n = 150)	04	06	16	11	11	07	23/150
Symptomatic (n = 42)	04	03	10	06	06	05	13/42
Total +/n	12/430 (2.8%)	20/430 (4.6%)	41/427 (9.6%)	26/429 (6.1%)	27/430 (6.3%)	13/22 (59.1%)	63/430 (14.6%)

BC = buffy coat; LN = lymph node; BM = bone marrow.
[a] Data published by Almeida et al. (2011).

Parasite isolation in culture is considered the gold standard in the diagnosis of infection by *Leishmania* spp., although this study found poor agreement between culture and PCR. However, the detection of DNA in all dogs with parasite isolation demonstrates reliability in the use of PCR as a diagnostic tool for CanL diagnosis. Variations in the results obtained in PCR may occur depending on the primers used and the target DNA amplified (Bastien et al., 2008). In our study, we used kinetoplastid DNA sequences that present large number of copies of the parasite, thus increasing the sensitivity of the test (Lachaud et al., 2002; Solcà et al., 2012).

PCR proved to be an efficient technique for detecting *Leishmania* infection; however, doubts regarding the best biological sample to be used encouraged many researches (Fisa et al., 2001; Lombardo et al., 2012; Manna et al., 2004; Teles et al., 2012). Most doubts refer to avoiding invasive procedures for the collection of biological samples. In this context, considering the high positivity rate for detection of *L. chagasi* DNA in lymph node aspirate compared to the others, samples analyzed highlight its use in epidemiological survey by PCR. Here, it is worth noting that several molecular approaches have been enhanced for the diagnosis of VL, such as real-time PCR, and recently, loop-mediated isothermal amplification has emerged as a promising test with many advantages over traditional PCR assays, mainly lower cost and facility of implementation in field setting (Khan et al., 2012).

Intact skin has also proved to be an excellent target for CanL diagnosis, mainly through culture (Almeida et al., 2012; Madeira et al.,

2009a), but cutaneous ulcers have also shown good results in the detection of *L. chagasi* DNA in this study. The assessment of this site is important for accurate diagnosis, particularly in overlapping areas of tegumentary and visceral leishmaniasis (Madeira et al., 2009a). Moreover, although other *Leishmania* spp. such as *L. braziliensis* and *L. amazonensis* were not previously detected in dogs in Cuiabá, this possibility should be considered in future studies in this region.

In CanL, depending on animal susceptibility, the parasites can be either eliminated resulting in self-limiting infection or disseminated to some sites causing generalized infection (Saridomichelakis, 2009). In this context, the biological sample, the diagnostic test used, and the immunological status of the animal must be considered. In our study, the likelihood of detecting *L. chagasi* DNA was higher in symptomatic animals when compared to those that presented slight signs or no clinical signs of infection. This result is in agreement with other reports, which showed that the clinical impairment of dogs facilitates CanL diagnosis, regardless of the test used (Martínez et al., 2011).

Cases of asymptomatic CanL represent more obstacles for accurate diagnosis, mainly because they constitute the clinical group with the highest prevalence in VL endemic areas (Marzochi et al., 2009), an aspect that was also observed in Cuiabá. Our results showed that symptomatic cases were easily diagnosed—using lymph node, bone marrow, and skin samples—when compared to asymptomatic dogs. Despite the fact that the asymptomatic dogs presented high positive results in buffy coat samples in this study, this datum was not significant, although the same sample shad already shown good results in other studies (Coura-Vital et al., 2011).

According to Maia et al. (2009), the lymph nodes, together with the spleen, are the preferred internal tissues for *L. chagasi* multiplication. Saridomichelakis (2009) also observed that the lymph nodes and the skin are the first sites to be in contact with the infection agent. Thus, parasitaemia in those organs can be constant in all the clinical phases of the infected dog, explaining the better performance of these samples in CanL diagnosis, regardless of the clinical manifestations, as also described by Teles et al. (2012).

In summary, the finding that the lymph node aspirate produced high positivity rates and the fact that this specimen was obtained by noninvasive methods highlight its use in epidemiological survey by PCR for CanL diagnosis.

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