SHORT REPORT: DETECTION OF LEISHMANIA DNA BY POLYMERASE CHAIN REACTION ON BLOOD SAMPLES FROM DOGS WITH VISCERAL LEISHMANIASIS

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Abstract. Immunological, parasitological, and molecular techniques were applied to blood samples of dogs to diagnose Leishmania infections. In 1997, 644 domestic dogs were studied. Peripheral blood samples were collected for serological diagnosis and detection of Leishmania parasite by polymerase chain reaction (PCR). The indirect immunofluorescence test was positive in 139 (21.6%) of 644 dogs examined. The PCR was performed in 70 blood samples and 3 bone marrow aspirates. A 120-bp fragment specific for Leishmania was present in PCR hybridization analysis of all seropositive samples in the molecular assays. The PCR hybridization test, which used a minicircle of Leishmania chagasi as a probe, was negative in 20 seronegative dogs. These results suggest that a combined PCR-Southern hybridization technique is a highly sensitive approach to diagnose leishmaniasis in dogs, which are a zoonotic reservoir of leishmaniasis for humans.

Visceral leishmaniasis (VL), or kala-azar, is a zoonotic disease. Dogs are the most important vertebrate reservoir, especially in areas where Leishmania chagasi or L. infantum are the causative agents of the disease. The prevalence of Leishmania infection in dogs in the American continent varies greatly. In Brazil, foci of VL have been reported in rural and suburban areas, including Minas Gerais State, occurring simultaneously in humans and dogs. The prevalence of canine VL is higher and normally precedes human disease.

Serologic screenings show that infected animals develop a specific antibody response, and the proportion of seroconversion among animals with subclinical or clinical infection is unknown. Methods of direct detection, which include Giemsa-stained smears of bone marrow, spleen, and liver, culture of specimens, and hamster inoculation confirm the disease in <80% of cases. This estimated sensitivity is often decreased as a result of culture contamination and the long incubation period in hamsters. Detection of parasites by microscopic examination of smears is dependent on the presence of a large number of parasites in the sample. The detection of parasite-specific antibodies in serum can be performed through indirect immunofluorescence test (IFT), enzyme-linked immunosorbent assay, or direct agglutination test. Healthy animals may be seropositive, and infected animals occasionally remain seronegative, hampering the use of such techniques.

Recently, several groups have shown that polymerase chain reaction (PCR) is a both sensitive and specific method for the detection of Leishmania DNA in a variety of clinical samples of human, dog, and fox. The PCR test was more sensitive than serology or culture assay in the diagnosis of canine VL in South America and Europe, suggesting that this methodology might become the gold standard for detecting Leishmania infections.

In the present study, we show the detection of Leishmania DNA by PCR and hybridization by use of kinetoplast DNA (kDNA) probes in canine blood samples. Comparison of the results obtained by IFT performed in serum from dogs of an endemic area for VL with the molecular data is presented.

The study was carried out with dogs kept by the Animal Protection Society of Minas Gerais. This society gives shelter to dogs neglected by their owners in the city of Belo Horizonte, Minas Gerais State, Brazil.

In 1997, 644 domestic mongrel dogs were studied. Peripheral blood samples were collected into disposable tubes containing ethylenediamine tetraacetic acid (EDTA) for serological and PCR analysis. In addition, bone marrow aspirates from seronegative (6 dogs) and serologically positive (10 dogs) were cultured in Novy, Nicolle & McNeal (NNN) blood agar medium submitted for direct observation and PCR. The blood sample was centrifuged, and the plasma and the cellular components were separately and stored at −20°C.

A commercial kit IFT for canine leishmaniasis diagnosis (FIocruz/Bio-Manguinhos) was performed for detection of antibodies in plasma diluted from 1:40 up to 1:2,560. A total of 70 blood samples and 3 bone marrow aspirates were used for the molecular analysis. Isolation of DNA was performed in 500 µL of whole blood by column chromatography (RapidPrep Micro Genomic DNA isolation kit for blood, Amersham Pharmacia Biotech Inc., Piscataway, NJ) following the manufacturer’s instructions. The DNA was further ethanol precipitated and resuspended in 10 µL TE (10 mM Tris·HCl, pH 8.0; 1 mM EDTA, pH 8.0). To amplify the conserved region of the minicircle molecule (kinetoplastid mitochondrial DNA; kDNA), oligonucleotides (primer A, 5'-G/C(G/C)(G/C)(G/C)(A/C) CTAT(A/T)TTTACCAAAACCC-3'; and primer B, 5'-GGGGAGGGGCGTCTGCGAA-3') were used in a hot-start PCR procedure. Briefly, each reaction contained 100 ng of 5' and 3' oligonucleotide primers, 200 µM of each deoxynucleoside triphosphate (Amersham Pharmacia, Uppsala, Sweden), 2.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT) in the buffer recommended by the manufacturer (1.5 mM MgCl2), and 2 µL of the DNA sample. We carried out PCR amplification in a DNA thermocycler (Perkin-Elmer) by use of the following cycles: 33 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, with a final cycle of 10 min at 72°C. Each experiment included a positive control (100 fg of Leishmania kDNA) and a negative control (no addition of DNA). The expected amplification products of 120 bp were analyzed by electrophoresis agarose gel containing ethidium bromide (0.5 µg/mL) at 100 V with 10 µL of the PCR reaction and visualized under ultraviolet light.
Detection of Leishmania DNA by PCR

Polymerase chain reaction (PCR) of clinical samples of blood and bone marrow aspirate of dogs with visceral leishmaniasis (VL). 2% agarose gel electrophoresis stained with ethidium bromide. MW 50 base-pair DNA ladder; DNA extracted from (lanes 01, 03, 05, 07) blood (lanes 02, 04, 06) bone marrow aspirate, positive control (kDNA extracted from cultured Leishmania chagasi promastigotes), and negative control (NC). B) Hybridization with a cloned minicircle from L. chagasi.

To avoid cross-contamination or PCR artifacts, a maximum of 4 samples each time underwent DNA extraction, which was performed in a separate and exclusive room. We prepared PCR mixtures in exclusive laminar flow hoods by use of reagents formed into aliquots, pipettes dedicated to PCR, and aerosol-resistant tips.

All PCR products were denatured in 0.4 N NaOH, applied to nylon membranes with a dot-blot apparatus, and hybridized with a cloned L. chagasi minicircle as a molecular probe radiolabeled with [α-32P] deoxyadenosine triphosphate (dATP) by random hexamer priming. Filters were hybridized at 65°C, washed in 0.1x sodium saline citrate at the same temperature, and exposed to X-ray film.

Of samples analyzed by IFT, 139 (21.6%) of the 644 dogs examined were positive, with titers varying from 1:40 to 1:2,560. None of the examined dogs had clinical manifestations of leishmaniasis.

We performed PCR on 70 blood samples (52 seropositive and 19 seronegative). Of the 52 seropositive samples that were submitted to the molecular tests, 39 (75%) presented the expected products in agarose gels (Figure 1). However, when the technique was coupled to molecular hybridization, samples that were negative by visual inspection of the agarose gels presented positive signals on film (52 of 52). Furthermore, a serologically negative dog (1 of 21) also presented a positive PCR hybridization result (Table 1).

Ten dogs with parasites in the bone marrow cultures, considered as positive controls, also had positive PCR hybridization in whole-blood samples, as well as in 3 positive bone marrow aspirates. Six serologically negative animals were used as negative controls because they were negative in all the techniques that were tested (Table 1).

American VL is a main public health problem in Brazil, where the prevalence of canine infection rates ranges 1–36% in different regions of the country. The high seroprevalence for VL in dogs presented herein (21.6%) indicates that canine VL is a problem of high magnitude in the urban area of Belo Horizonte. The main limitation for serological study is its low specificity: Trypanosoma cruzi and Leishmania (Viannia) braziliensis, which shares many common epitopes with Leishmania chagasi, are particularly common parasites in Minas Gerais State, leading to a false-positive result.22,23

On the other hand, false-negative findings may occur, either because of a delay in the prepatent period or because some dogs never seroconvert.24 Therefore, a more sensitive and specific method is needed to confirm the infection.

In this study, we found that the PCR hybridization technique was very sensitive; all of the 10 dogs proven to be infected presented the parasite DNA by this technique, similar to what has been described by others.11,12,18,25 Weiber and others26 reported an 87% positivity in blood and 100% with bone marrow, thus indicating the usefulness of PCR for both diagnosis and follow-up treatment of canine leishmaniasis. Indeed, PCR is known to be both specific and sensitive. However, the technique requires a well-equipped laboratory and trained staff in order to avoid cross-contamination of samples. False-positive findings caused by laboratory contamination are the main limitation of PCR diagnosis.11 However, this occurrence in our study seems unlikely because extensive precautions were taken, and all negative controls of the PCR reactions, as well as the 6 animals found to be negative by serology and bone marrow culture, were always negative by hybridization. Therefore, the PCR methodology described herein, coupled with hybridization, which allows the identification of the parasite species, is useful for the diagnosis of VL. Our approach has promise and may lead to a better understanding of the epidemiology of leishmaniasis in an area that is endemic for both VL and cutaneous leishmaniasis.

Results of different techniques for diagnosing canine leishmaniasis in 73 dogs

<table>
<thead>
<tr>
<th>Technique</th>
<th>Positive IFT (%) (n = 52)</th>
<th>Negative IFT (%) (n = 21)</th>
<th>Negative control (%) (n = 6)</th>
<th>Positive control (%) (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serology (IFT)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Hybridization</td>
<td>100</td>
<td>4.8</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*IFT = immunofluorescence test.

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