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COMPARISON OF CLINICAL SAMPLES FOR VISCERAL LEISHMANIASIS DIAGNOSIS IN ASYMPTOMATIC DOGS BY PCR-HYBRIDIZATION

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ABSTRACT

The canine visceral leishmaniasis (CVL) diagnosis still represents a challenge because of complexity of this disease. The aim of present study was to compare different clinical samples for diagnosis of CVL by Polymerase Chain Reaction (PCR) combined with hybridization of ^{32}P labeled probes. Bone marrow (BM), skin biopsy (SB), peripheral blood (PB) and conjunctival swab (CS) were used in this work. With this purpose 40 asymptomatic dogs, all positive by parasitological test, were obtained. From each animal were collected SB with sterile punches from ear internal surface, 1.0 mL of PB, BM aspirates from sternum and CS from both lower eyelid. Each clinical sample was submitted to suitable DNA purification process and PCR-hybridization assays. The positive results obtained with PCR were 55%, 25%, 30% and 22.5% for CS, BM, SB and PB respectively while the PCR followed by hybridization showed a positivity of 87.5%, 50%, 45% and 27.5% respectively. The hybridization assay was able to increase the PCR positivity in all kinds of clinical samples. The best performance was obtained using CS samples. We concluded that the PCR associated with DNA radioactive probes was a very sensitive tool for diagnosis of CVL in asymptomatic dogs and the CS has an important potential for regular screening of dogs.

1. INTRODUCTION

The leishmaniasis is an infectious disease transmitted by the bite of phlebotomine sand flies and shows a wide clinical spectrum in humans. The visceral leishmaniasis (VL) is the most severe manifestation and is caused by species belonged to *Leishmania (Leishmania) donovani* complex. In New World the VL is a zoonotic disease and *L. (L.) chagasi* (=*L. infantum*) is the etiological agent being Brazil the most affected country in Latin America [1]. The dogs are the main domestic reservoir of VL because they are very susceptible and have a high parasitism in the skin [2]. The canine VL (CVL) prevalence is usually high in Brazilian endemic regions and a lot of infected dogs do not reveal any clinical signs. However, these animals have a great epidemiological importance because they are able to infect phlebotomines [3]. In addition, it has been shown that CVL is a risk factor for human leishmaniasis once canine infections can precede the human ones [4]. According to the World

Health Organization, the main control measures for VL are: diagnostic and treatment of human cases, insecticide vector control and *Leishmania*-seropositive dog sacrifice. So, the CVL diagnosis becomes an essential step in disease control. The serodiagnosis is used in large-scale in Brazil for screening dogs mainly by Enzyme Linked Immunosorbent Assay (ELISA) and Immunofluorescence Antibody Test (IFAT). In these tests, specific antibodies against *Leishmania* are detected in the host's serum. Nevertheless, it has been demonstrated these methodologies underestimate the number of infected animals due to high number of dogs with low level of specific antibodies and difficulty to detect the infection in asymptomatic dogs [5]. The parasitological test involves direct detection of parasites and has been considered a gold standard for diagnosis because, with a positive result, there is no doubt about the presence of infection. On the other hand, this test requires invasive sampling methods and has low sensitivity [6]. The Polymerase Chain Reaction (PCR) has been pointed as a highly sensitive and specific diagnostic test for leishmaniasis [3]. In this case, specific genetic sequences of parasites are amplified and detected from different kinds of clinical samples as peripheral blood (PB), bone marrow (BM), skin, blood spotted onto filter paper, spleen, lymph node and conjunctival swab (CS) [4], [7]. The hybridization of the PCR products with radiolabeled probes is an interesting complement technique because it confirms the PCR results and increases the sensitivity [7]. The aim of this work was to compare different clinical samples as BM, PB, skin biopsies (SB) and CS for CVL diagnosis by PCR-hybridization.

2. MATERIAL AND METHODS

2.1) Dogs and samples

This project was previously approved by Ethical Committee in Animal Experimentation from "Universidade Federal de Minas Gerais" (protocol number: 183/08). Forty seropositive dogs by ELISA and IFAT tests were obtained from the Municipal Zoonotic Diseases Control Department from Belo Horizonte. They presented a positive parasitological test and were clinically evaluated as asymptomatic. From each dog 1.0mL of PB was collected in tubes containing EDTA, a SB was obtained with 5.0mm sterile punch from ear internal surface and BM aspirates were collected from sternum. Besides, a CS was carried out in lower conjunctive in both eyes from all animals using sterile cotton swabs manufactured for bacteriological isolation.

2.2) Parasitological test

For parasitological confirmation, a fraction from BM aspirates was inoculated in biphasic medium (Novy-McCneal-Nicolle and α-Minimum Essential Medium). In addition, slide smears were prepared from SB and BM samples. These materials were stained by fast *panótico* (Bioclin do Brasil)™ according to the manufacturers and analyzed by optical microscopy.

2.3) DNA extraction

The DNA was purified from PB using the GE Healthcare Illustra™ Blood Genomic Prep Mini Spin kit. From SB and BM, the DNA was extracted using the Wizard™ - SV Genomic

DNA Purification System – Promega and GE Healthcare Illustra™ Blood Genomic Prep Mini Spin kit respectively. Finally, the cotton swabs used for CS were submitted to phenol-chloroform method for DNA purification [7]. All DNA preparations were stored at -20°C until their use for PCR assays.

2.4) PCR

The PCR mixture contained 1.0μL of DNA preparation, 0.2mM each of dNTP, 200ng of each primer [5'–(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACAACCCC-3' and 5'GGGGAGGGGCGTTCTGCGAA-3'], 2.5U of Taq polymerase (Ludwig Biotech™), 2.5μL 10X buffer (Tris-HCl 50mM, [pH 8.3], KCl 50mM), 2.0mM $MgCl_2$ in a final volume of 25μL. Positive controls with *L. (L.) chagasi* (strain MHOM/1973/BH-46) and *L.(Viannia) braziliensis* (strain MHOM/1975/BR/ M2903) genomic DNA were used with a final concentration of 1.0ng/μL. Furthermore, DNA from CS of a dog already known as infected animal was used either. As negative controls preparations without DNA and DNA purified from CS of a healthy negative dog for leishmaniasis were included in all tests. All amplification products were analyzed on 2% agarose gel and were stained with ethidium bromide. The target of amplification was a 120 base pairs (bp) sequence of kinetoplast DNA (kDNA) mini-circle conserved region.

2.5) Dot Blot

10μL of each amplification product were applied on a nylon membrane in a Bio Dot apparatus. The filter received 111μL of NaOH 0.4M, EDTA 25mM [pH 8.0] solution and then was rinsed with 2X SSC (0.3M NaCl, 0.3mM sodium citrate) and dried. The DNA was fixed to the filter through the UV light (0.12J/cm^2).

2.6) Hybridization

Cloned kDNA mini-circles from *L. (L.) chagasi* were labeled with ^{32}P[α]dCTP using the Random Primer method (Invitrogen™). The filters were pre-soaked at 58°C for 30min in 0.5% non-fat milk, 1% sodium dodecyl sulphate (SDS) and 2X SSC solution. The kDNA probe was added to the solution after being heated for 3min in a boiling water bath. The filter was incubated for 14h at 58°C, with shaking, then placed in 2X SSC at room temperature and washed in 0.5X SSC, 0.5X SDS, at 65°C for 30min. Finally it was dried and exposed to autoradiography in *Bio-Imaging Analyzer* (Fujifilm™) set for 2h. After that the same membrane was washed with 0.1X SSC and boiled during 5 minutes for removing the probes. Another hybridization was made using *L. (V.) braziliensis* cloned kDNA mini-circles probes. The procedures were carried out as described for *L. (L.) chagasi* probes.

3. RESULTS

The percentages of positive results for PCR were 55%, 25%, 30% and 22.5% for CS, BM, SB and PB respectively. The positivities obtained by PCR followed by hybridization were superior to the last ones: 87.5%, 50%, 45% and 27.5% respectively. In this case, the data for CS were calculated combining samples of both eyes (Fig. 1).

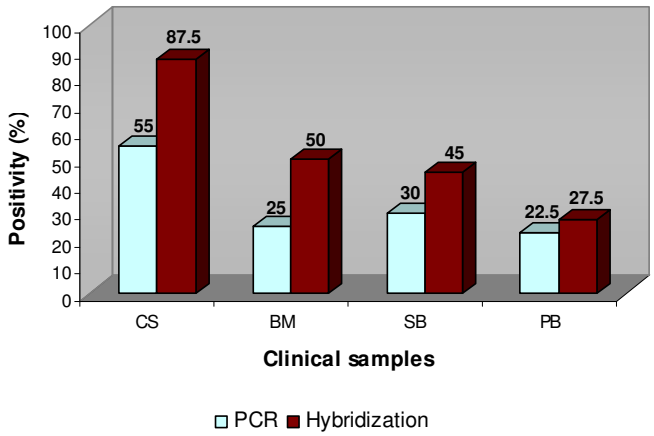

Figure 1: Positivities obtained by PCR and PCR followed by hybridization for different clinical samples in 40 asymptomatic dogs. The CS data were calculated associating both conjunctivas. Numbers above bars indicate the percentage of positive samples. CS = Conjunctival swab; BM = Bone marrow; SB = Skin biopsy; PB = Peripheral blood.

The figures 2-A and 2-B show a combination of a representative electrophoresis gel for CS samples and their respective results for hybridization. In the hybridization assays all dogs were found infected by *L. (L.) chagasi.*

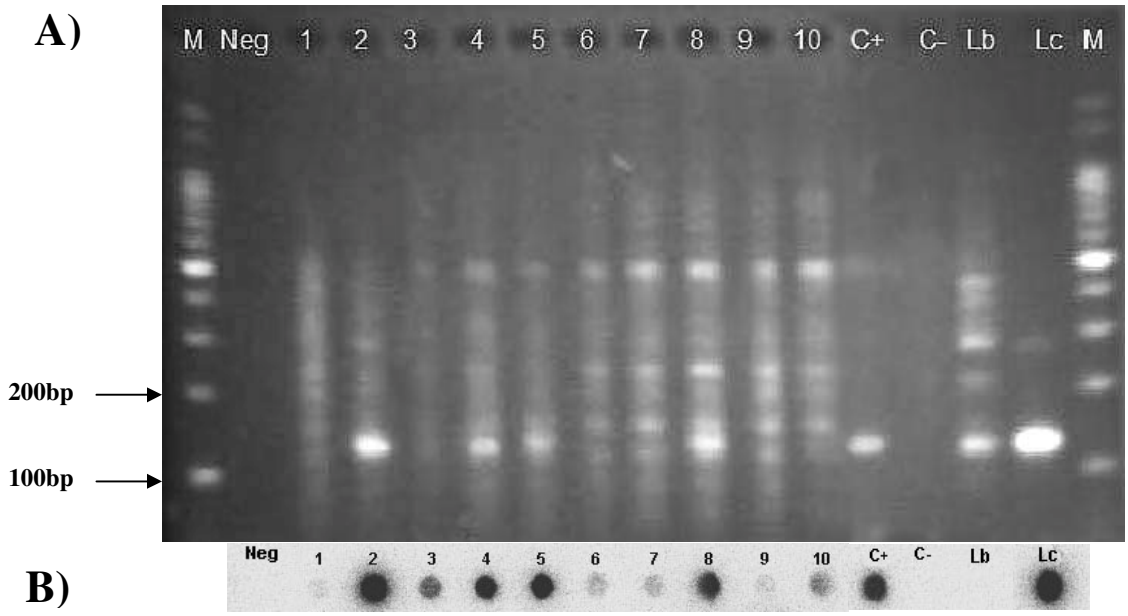

Figure 2. (A): Agarose gel of PCR products for CS of right conjunctive. (B): Autoradiogram of the same sample. In this membrane *L. (L.) chagasi* probes were used. M: Molecular weight marker; Neg: Negative control without DNA; 1 to 10: Dogs' codes; C+: Positive control from a dog already known as infected with *Leishmania*; C-: Negative control from a healthy dog without infection; Lb: Positive control from *L. (V.) braziliensis*; Lc: Positive control from *L. (L.) chagasi*.

4. CONCLUSION

According to the results of this work the hybridization increased the PCR positivity in all kinds of clinical samples used. This reinforces the usefulness of radiolabeled DNA probes associated with PCR for leishmaniasis molecular diagnosis. An efficient diagnosis of CVL requires the correct detection of infected asymptomatic dogs because they have a great epidemiological importance and represent a big proportion of all animals with infection. In this study, a high positivity was obtained with CS material. So, we conclude that the combination of these molecular techniques with the CS samples permitted a good sensitivity for CVL diagnosis and can represent an important auxiliary tool for screening dogs.

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