2011 International Nuclear Atlantic Conference - INAC 2011
Belo Horizonte,MG, Brazil, October 24-28, 2011
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-04-5**

Diagnosis of canine visceral leishmaniasis with radiolabelled probes: Comparison of the kDNA PCR-hybridization with three molecular methods in different clinical samples.

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ABSTRACT

Leishmania (Leishmania) chagasi is responsible for visceral leishmaniasis (VL) in Brazil and the dog is the main domestic reservoir. Disease control is based on the elimination of infected animals and the use of a sensitive and specific diagnostic test is necessary. The Brazilian VL control program emphasizes serologic surveys, mainly using the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT), followed by the elimination of the seropositive dogs. However, these techniques present limitations in terms of sensitivity and specificity. The Polymerase Chain Reaction (PCR) associated to hybridization with DNA probes labeled with ^{32}P has been recognized as a valuable tool for *Leishmania* identification. In this study, the sensitivity of kDNA PCR hybridization method was compared with three other molecular methods: Internal Transcribed Spacer 1 Nested PCR (ITS-1nPCR), *Leishmania* nested PCR (LnPCR) and Semi-nested kDNA PCR (kDNA snPCR). The comparison was performed in different clinical specimens: conjunctival swab, skin, blood and bone marrow. A group of thirty symptomatic dogs, positive in the parasitological and serological tests, was used. When. The techniques targeting kDNA mini-circles (kDNA snPCR and KDNA PCR-hybridization) showed the worst result for blood samples. The KDNA-PCR hybridization showed the best sensitivity for conjunctival swab. By comparing the samples on the basis of positivity obtained by the sum of all methods, the blood showed the worst outcome (71/120).The bone marrow showed the highest positivity (106/120), followed by conjunctival swab (100/120) and skin (89/120). Since the bone marrow samples are unsuitable for routine epidemiological surveys, the conjunctival swab was recommended because it allows high sensitivity, especially when associated with kDNA PCR hybridization method, and is a noninvasive sampling method.

Keywords: PCR, canine visceral leishmaniasis, diagnosis, radioactive DNA probe

1. INTRODUCTION

Visceral leishmaniasis (VL) is an infectious parasitic disease, commonly described as the most severe form of leishmaniasis. The disease is potentially fatal if untreated [9]. In Brazil, the *Leishmania (Leishmania) infantum (= L.(L.) chagasi),* associated to the vector *Lutzomyia longipalpis* has frequently caused the disease. The Brazilian Ministry of Health suggests three measures to control the disease: the early treatment of infected persons, vector control and elimination of infected dogs. Currently, the cities of Belo Horizonte,

Montes Claros, Ribeirão das Neves, Janaúba, Santa Luzia and Paracatu are responsible for 56% of leishmaniasis cases in Minas Gerais [11].

The dogs act as reservoir and often harbor the parasite in the dermis. These hosts take part directly on the biological cycle of the disease transmission, since they act as a source of infection for the vector during the blood meal [13 and 14]. As the disease control involves the elimination of infected animals, the correct diagnosis is necessary to prevent disease transmission or unnecessary sacrifice of dogs. Current diagnostic methods are based on serology, mainly the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT). However, these tests routinely demonstrated problems of specificity and sensitivity. The PCR technique associated with radioactive probes is an alternative in this scenario, since the DNA of the parasite is the direct target of the analysis. One of the biggest obstacles in the implementation of molecular assays is the lack of standardization. More than 400 publications on the molecular diagnosis of leishmaniasis have been held since 1989, but few compare the efficiency of several available techniques.

The kDNA has been the target of choice to identify *Leishmania* in many laboratories. It consists of maxicircles and minicircles. The minicircles measuring about 1 kb are present in 10,000 to 20,000 copies per cell. The minicircles of *Leishmania* are almost ideal as molecular targets, because they are present in high amount and contain a conserved region of about 120 bp [20].

The PCR associated with DNA probes hybridization allowed accurate results in leishmaniasis diagnosis studies [12]. In this work the PCR method used is based on the amplification of kDNA minicircles conserved region followed by PCR products hybridization with cloned *L. chagasi* minicircle probes labeled with ^{32}P [7].

The hybridization with minicircle probes allows the identification of *Leishmania* subgenera, fact already demonstrated in early studies where the use of kDNA probes allowed the distinction between the complexes *L. braziliensis* and *L. Mexicana* [6]. The secondary detection using specific probes for PCR products identification is highly desirable, since it increases the specificity and the sensitivity (by 10 times) [10].

In this study the value of four sensitive and accurate PCR methods used for *Leishmania* detection were compared in samples of blood, skin biopsy, bone marrow and conjunctival swab. Two of the methods used primers addressed to kinetoplast minicircles and the other two to rRNA ribossomal genes.

2. MATERIAL AND METHODS

2.1. Dogs and samples

Thirty symptomatic mongrel dogs positives in the serology and the parasitological exams were used in this study. Six dogs from a non endemic area were submitted to the same procedure and used as negative controls. The DNA extraction from the swabs were performed by phenol-chloroform method. Commercial kits were used for DNA extraction of samples of blood, bone marrow and skin biopsies. This work was approved by the Animal Experimentation Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG) protocol number 198/2006.

2.2. PCR analysis

Positive controls with *L. (L.) chagasi* (strain MHOM/1973/BH46) genomic DNA were used at 1.0 ng/µl. A negative control without DNA was included in all tests. The DNA sample volume of 1.0 µl was used for all methods. Four PCR protocols were tested.

2.2.1. kDNA PCR- hybridization

The PCR reaction mixture contained 0.2 mM of each dNTP, 0.2 nmol of each primer [5'–(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACAACCCC-3' and 5'GGGGAGGGGCGTTCTGCGAA-3'], 2.5 U of AmpliTaq Gold® (Applied Biosystems), 5.0 µl of 10X buffer (Tris-HCl 50 mM, [pH 8.3], KCl 50 mM), 2.0 mM $MgCl_2$ in a final volume of 50 µl. The amplifying conditions were: initial denaturation at 95°C for 15 min, 30 cycles of 94°C at 30 s, 50°C at 30 s, 72°C at 30 s, followed by a final extension at 72°C for 10min. The target for amplification was a 120 base pairs (bp) sequence of *Leishmania* kDNA minicircle conserved region [7]. For the hybridization step 10 µl of each amplification product was mixed with 110 µl of NaOH 0.4 M, EDTA 25 mM [pH 8.0] solution and spotted on a nylon membrane. Cloned kDNA minicircles from *L. (L.) chagasi* were used as probes. The probe was labeled with $^{32}P[\alpha]dCTP$ using the Random Primer DNA Labeling System (Invitrogen). Hybridization conditions were as previously described [1].

2.2.2. kDNA snPCR (seminested PCR)

The seminested reaction was performed as previously described [16] with minor modifications. For the first amplification 1.0 µl of DNA solution was added to 49µl of PCR mix containing 15 pmol specific *Leishmania* primers DRJ, 5´-CGA TTT TTG AAC GGG ATT TCT GCA C-3´, and KLK2, 5´-CTC CGG GGC GGG AAA CTG G-3´; 0.2 mM dNTP; 2mM $MgCl_2$; 5 mM KCl; 75 mM Tris HCl (pH 9.0); 2.0 mM $(NH_4)SO_4$ and 1,4 U of Taq DNA polymerase (Ludwig Biotec). The following cycling conditions were used: 94°C for 5 min, 30 cycles at 94°C for 30 s and 67°C for 30 s, with an extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplification product of 800 bp is related to the variable region of kDNA minicircles. For the reaction of reamplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification, with 7.5 pmol DRJ primer, 7.5 pmol of a new *Leishmania* specific primer AJS31, 5´-GGG GTT GGT GTA AAA TAG GGC CGG-3´, and 0.7 U of Taq DNA polymerase (Ludwig Biotec). Positive samples yielded a PCR product of 780 bp.

2.2.3. ITS-1 nPCR (internal transcribed spacer 1 nested PCR)

This method was adapted from [22]. Primers addressed to internal transcribed spacer 1 (ITS 1) between the genes coding for SSU rRNA and 5.8S rRNA were used. For the first amplification 1.0 µl of DNA solution was added to 49 µl of PCR mix containing 15 pmol of the primers 5´- CTGGATCATTTTCCGATG-3´ and 5´- TGATACCACTTATCGCACTT-3´ and 0.2 mM deoxynucleoside triphosphates, 2mM $MgCl_2$, 5 mM KCl, 75 mM Tris-HCl pH 9.0, 2.0 mM (NH_4) SO_4, and 1.4 U of Taq DNA polymerase (Ludwig Biotec). The cycling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The PCR product size

expected lies between 300 and 350 bp. For the second amplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification but with the following primers (15 pmol each): 5´-CATTTTCCGATGATTACACC-3´ and 5´-CGTTCTTCAACGAAATAGG-3´. Positive samples yielded a PCR product between 280 and 330 bp.

2.2.4. LnPCR (*Leishmania* nested PCR)

This method uses primers addressed to the variable region of SSUrRNA (small subunit ribosomal ribonucleic acid) gene and was performed according [5] with minor modifications. For the first amplification 1.0 µl of DNA solution was added to 49µl of PCR mix containing 15 pmol of the Kinetoplastida-specific primes 5´-GGTTCCTTTCCTGATTTACG-3´ and 5´-GGCCGGTAAAGGCCGAATAG-3´, 0.2 mM deoxynucleoside triphosphates, 2mM $MgCl_2$, 5 mM KCl, 75 mM Tris-HCl pH 9.0, 2.0 mM $(NH_4)SO_4$, and 1.4 U of Taq DNA polymerase (Ludwig Biotec). The cycling conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. Samples revealing a 603 bp product were scored as positive. For the second amplification 10 µl of a 1:40 dilution of the first PCR product was added to 15 µl of PCR mix under the same conditions as the first amplification but with the following *Leishmania*-specific primers (15 pmol each): 5´-TCCCATCGCAACCTCGGTT-3´ and R333 5´-AAAGCGGGCGCGGTGCTG-3´and 0.7 U of Taq DNA polymerase (Ludwig Biotec) using an annealing temperature of 65°C in the cycles. Positive samples yielded a PCR product of 353 bp.

2.3. Statistical analysis

The results were analyzed using the Pearson chi square test. The difference between the results was considered significant with P value < 0.05.

3. RESULTS AND DISCUSSION

The percentage of positive dogs in each group of samples was compared between the examined methods (Figure 1). The methods that target the kDNA minicircle (kDNA snPCR and kDNA PCR hybridization) showed a poor performance in blood samples No statistical difference was found between methods for samples of skin and bone marrow. The kDNA PCR hybridization in the conjunctival swab samples presented a higher and significant difference in relation to kDNA snPCR and LnPCR.

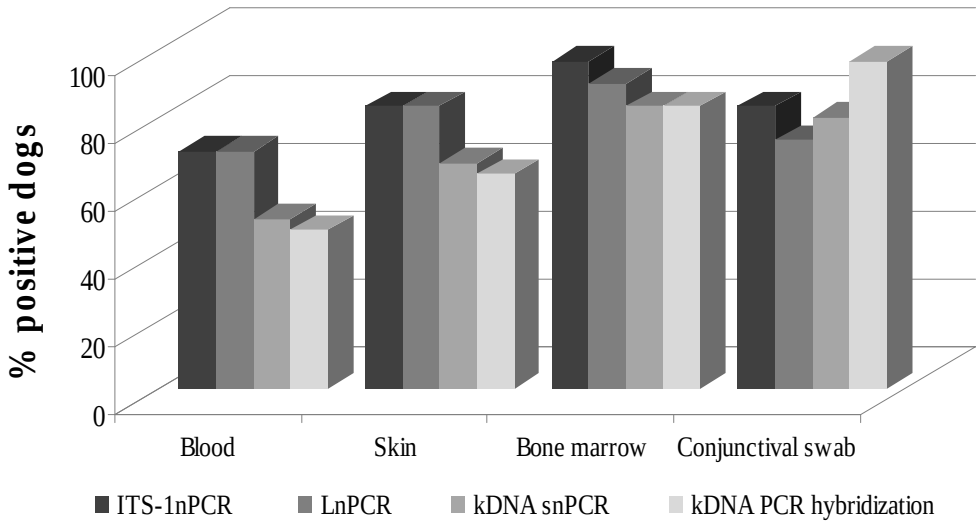

Figure 1. Comparison of PCR methods in four clinical samples.

The total positivity in each of the techniques was obtained from the sum of all clinical samples, as shown in Figure 2. Significant difference was found between ITS-1 nPCR in relation to kDNA snPCR and kDNA PCR hybridization.

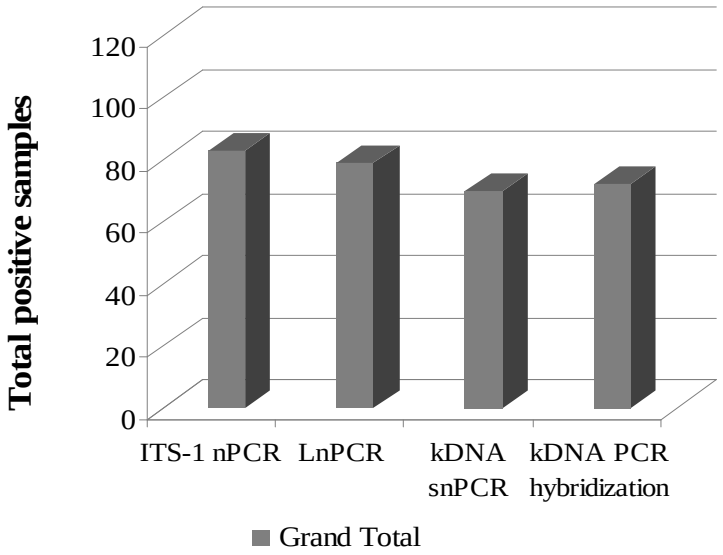

Figure 2. Total positivity for each method obtained by the sum of all clinical samples.

The total number of positive samples for each group of clinical sample obtained by the sum of all methods was showed in the figure 3. Blood samples showed the worst performance significantly lower in relation to all other samples. Statistical differences were also verified between the results of skin and bone marrow. However, there was no difference between the skin and conjunctival swab, as well as between bone marrow and conjunctival swab.

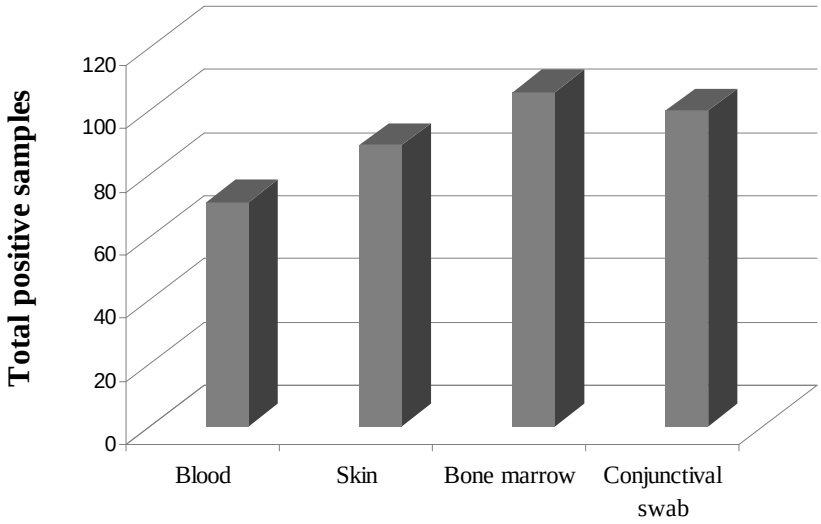

Figure 3. Total positivity obtained for each clinical sample obtained by the sum of all methods.

4. CONCLUSIONS

- The methods that target the kDNA showed lower positivity in blood samples;
- Blood samples presented the lower sensitivity for all PCR methods evaluated;
- The kDNA PCR hybridization showed high positivity in conjunctival swab samples, statistically higher in relation to LnPCR and KDNA snPCR;
- The ITS-1 nPCR method showed the best result when comparing the sum of positivities of all clinical samples;
- Bone marrow showed the highest positivity followed by conjunctival swab when comparing the sum of positive results of all samples;
- The conjunctival swab could be used for routine epidemiological surveys since it is a noninvasive sample that allows a high sensitivity;
- The results of this study support the the conjunctival swab applicability for the diagnosis of canine visceral leishmaniasis.

ACKNOWLEDGMENTS

This project was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (470386/2010-0), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (CBB-APQ 00339-10) and Comissão Nacional de Energia Nuclear (CNEN).

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