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DIAGNOSIS OF VISCERAL LEISHMANIASIS IN ASYMPTOMATIC DOGS BY THE KDNA PCR-HYBRIDIZATION ASSAY USING NON-INVASIVE SAMPLES

Rodrigo Souza Leite[1], Sydney de Almeida Ferreira[2], Leonardo Trindade Ituassu[2], Maria Norma de Melo[2] and Antero Silva Ribeiro de Andrade[1]

[1] Laboratório de Radiobiologia – Centro de Desenvolvimento da Tecnologia Nuclear, CDTN
Avenida Presidente Antônio Carlos, 6.627, Campus da UFMG
CEP 31270-901 - Belo Horizonte, MG
rleite2005@gmail.com

[2] Departamento de Parasitologia – Instituto de Ciências Biológicas, ICB
Universidade Federal de Minas Gerais
Avenida Presidente Antônio Carlos, 6.627, Campus da UFMG
Caixa Postal 486
31270-901, Belo Horizonte, MG
saninoalmeida@gmail.com

ABSTRACT

The visceral leishmaniasis (VL) in Brazil is caused by *Leishmania (Leishmania) chagas*i and the asymptomatic dogs may transmit the parasite to sand flies vectors. The VL epidemiological control in Brazil involves the elimination of seropositive dogs, insecticide treatment and systematic treatment of human cases. Therefore, the accurate diagnosis is important in order to avoid the disease transmission or unnecessary culling of dogs. Serological tests are used for screening of dogs. However, these techniques present limitations. The Polymerase Chain Reaction (PCR) is an attractive alternative to the diagnosis in this context; but non-invasive samplings have great importance because they are simpler, painless and less resisted by dog-owners. This study aimed at evaluating conjunctival swab (CS) for canine VL diagnosis. In this methodology a sterile cotton swab is used to sampling the dog conjunctiva in both eyes. Thirty asymptomatic seropositive dogs were used. The samples were analyzed by the kDNA PCR–hybridization procedure in which the PCR products are hybridized with cloned kDNA mini-circles labeled with ^{32}P[α]dCTP. In addition, blood (B) was collected from each animal. *L. chagasi* was identified in 90% of CS samples and 13,6% of B samples. The high sensitivity obtained with asymptomatic dogs, in which the diagnosis is more difficult due the low number of parasites in the samples, allow concluding that the conjunctival swab associated to the kDNA PCR-hybridization assay provides a valuable alternative tool for the direct diagnosis of canine leishmaniasis.

1. INTRODUCTION

Nowadays, leishmaniasis is a serious problem of public health and affects around 12 million of people in the world [1]. Visceral leishmaniasis (VL) is considered the most lethal form of the disease and 90% of the cases occur in only a few countries: Bangladesh, India, Sudan and Brazil [2]. VL in Latin America is caused by *Leishmania (Leishmania) chagasi* and Brazil reports most of the cases in this area [3].

Dogs are considered the main domestic reservoir of the parasite, constituting part of the epidemiological cycle of human transmission [4], with Brazil accounting for more than 90% of VL cases described in the New World [5]

In endemic areas serological tests are used for diagnosis and also as tools in epidemiological studies, the most employed techniques being indirect immunofluorescence [6] and ELISA [7]. However, these techniques present limitations in terms of reproducibility and specificity[8]. Sometimes they fail due the low levels of specific antibodies in some dogs and cross-reactivities with Chagas's disease, rickettsiosis and toxoplasmosis [9].

According to the Zoonotic Disease Control Center of Belo Horizonte, leishmaniasis profile has changed in latest years, since the number of asymptomatic dogs are increasing. In this animals the diagnostic is more difficult given that parasite charge is low. Therefore, new tools for disease diagnosis are necessary.

The Polymerase Chain Reaction (PCR) is an attractive alternative for diagnosis in asymptomatic dogs since it detects the parasite's DNA and not the host's antibodies. Studies have shown the PCR as a method both highly specific and more sensitive than the classical procedures for canine VL diagnosis [10] Several kinds of biological samples can be used for the PCR, such as: spleen, lymph nodes, skin, conjunctival biopsy, blood and bone marrow. Blood and bone marrow are usually the most common tissues used [11]. The non-invasive samplings assume great importance in this context because they are simpler, painless and more easily allowed by the dog-owners. An interesting approach in this context is the conjunctival swab (CS) procedure, a method for sample collection that uses a sterile swab for sampling the dog conjunctivas. This method was shown to be highly sensitive when used for diagnosis of symptomatic dogs [10; 12; 13]. The aim of the present study was to evaluate the conjunctival swab sampling method for canine VL diagnosis by PCR in asymptomatic dogs. The samples were analyzed by the PCR-hybridization assay, in which the PCR products are further hybridized with radioactive DNA probes.

2. MATERIAL AND METHODS

2.1. Animals and samples

Thirty asymptomatic seropositive dogs, destined for euthanasia, were used. Animals were provided for the Municipal Zoonotic Diseases Control Department of Belo Horizonte. The dogs were submitted to clinical examination and only asymptomatic dogs simultaneously positive for ELISA, IFAT were used in this study. Exfoliative epithelial cells were collected from the right (RC) and left (LC) conjunctivas of each animal using sterile cotton swabs manufactured for bacteriological isolation (Fig. 1). Each sample was analyzed separately to provide a replicate diagnosis for each animal. The cotton tips were broken and only the cotton parts were transferred to sterile tubes and stored at - 20°C until being used. Blood (1.0 ml) was collected from each dog in EDTA containing tubes, kept in ice and processed as soon as possible. Bone Marrow (BM) was collected from each dog for parasitological diagnostic. The results were compared by evaluating the frequency of positivity of CS samples in relation to those obtained from blood samples. This work was approved by the Animal Experimentation Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG) protocol number198/2006.

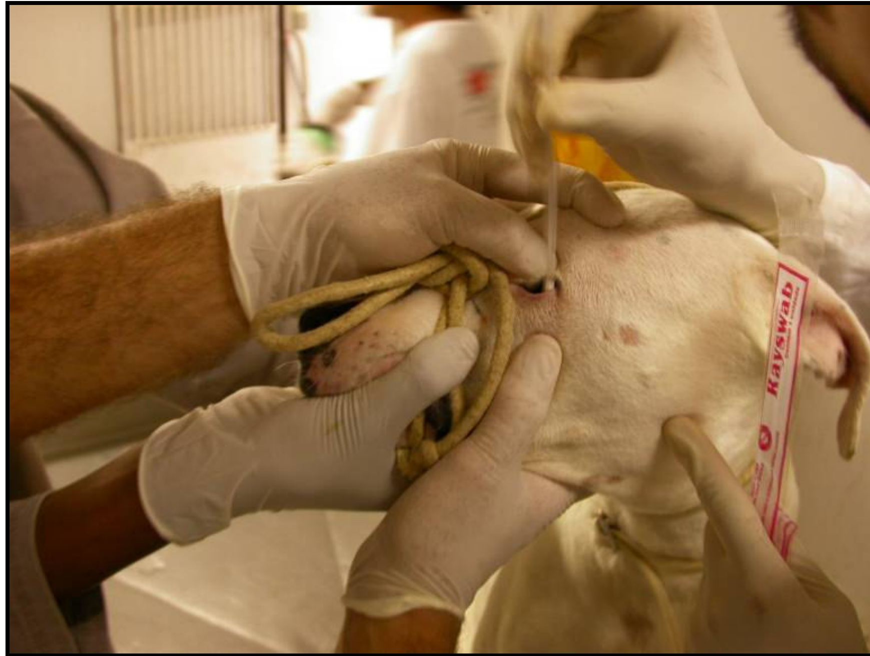

Figure 1: Sample collection using cotton swab. The swabs were rubbed against the surface of the lower eyelid to collect the cells. The conjunctivas of both eyes were sampled.

2.2 DNA purification

The DNA purification from CS was carried out by the phenol–chloroform–isoamyl alcohol method as previously described [10] with minor modifications. Each cotton received 300 µl of lysis buffer (50 mmol/l Tris, 50 mmol/l NaCl, and 10 mmol/l EDTA, pH 8.0) containing proteinase K (250 mg/ml) and Triton X-100 (1%). After the incubation (2 h at 56°C) the solution was eluted from the cotton, transferred into phase-lock gel tube PLG-H (Eppendorf) and was mixed with 500 ml of 75% Tris–saturated phenol (Sigma), 25% chloroform–isoamyl alcohol. The organic phase was separated from the aqueous phase by centrifugation at 12,000 x *g* for 5 min. The aqueous phase was transferred to a new phase-lock gel tube PLG-H. The extraction was repeated with 500 ml of 50% phenol, 50% chloroform isoamyl alcohol and once with 100% chloroform-isoamyl alcohol. The DNA precipitation was done with one volume of isopropanol–sodium acetate, followed by wash with 75% ethanol. The DNA pellet was suspended in 30 ml of deionized water and was kept at 4-8C until being used.
The DNA extraction from total blood was performed using the illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare) according to the manufacturer's protocol.

2.3 PCR

The PCR reaction mixture contained 1.0 µl of DNA solution, 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 Taq DNA polymerase (Ludwig Biotec), 2.5 µl of 10 x buffer (Tris–HCl 50 mM, [pH 8.3], KCl 50 mM), 2.0 mM $MgCl_2$ in a final

volume of 25 ml. 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 amplifying conditions were: initial denaturation at 95°C for 15 min, 30 cycles including 94°C at 30s, 50°C at 30 s, 72°C at 30 s, and a final extension at 72°C for 10 min. All amplification products were analyzed on 2% agarose gel and were stained with ethidium bromide (5 mg/ml). The target for amplification was a 120 base pairs (bp) sequence of *Leishmania* kDNA mini-circle conserved region [14; 15].

2.4 Hybridization

A total of 10 µl of each amplification product was mixed to 110 ml of NaOH 0.4 M, EDTA 25 mM [pH 8.0] solution and spotted on nylon membrane (Hybond -XL Amersham) using a Bio Dot apparatus (Hybrid-dot manifold-BRL1). After that, the membrane was rinsed with 2 x SSC (0.3 MNaCl, 0.3 mM sodium citrate) and dried. The DNA was fixed to the filter by UV light (0.12 J/cm2). Cloned kDNA mini-circles from *L. (L.) chagasi* were used as probes. The probe was labeled with ^{32}P[α]dCTP using the Random Primer DNA Labeling System (Invitrogen). Hybridization conditions were as previously described [15]. Briefly, the filters were pre-soaked at 58°C for 30 min in 0.5% non fat milk, 1% sodium dodecyl sulphate (SDS) and 2 x SSC solution. The kDNA probe was added to the solution after being heated for 3 min in a boiling water bath. The filters were incubated for 14 h at 58°C, with shaking, then placed in 2 x SSC at room temperature for 20 min and washed in 0.5 x SSC, 0.5 x SDS, at 65°C for 30min. Finally, it was dried and exposed in the cassette (BAS 2325 Fujifilm) for 2 h. The image (Fig. 2)was obtained using a *Bio-Imaging Analyzer* (Fujifilm).

3. RESULTS

Table 1 summarizes the PCR results of B and CS from dogs affected by *Leishmania* infection. Using the kDNA PCR, 18 of 30 dogs (45%) were positive for CS samples while for B samples the positivity was 0%.

Table 1. Number of positive samples in kDNA PCR without Hybridization

Sample	Number of positive
CS	18 (45%)
B	0

After hybridization, CS and B samples showed a significant increase in the positivity (Table 2). After hybridization with DNA probe labeled with ^{32}P[α]dCTP, CS samples were positive in 27 of 30 animals (90%) and B samples in 4 of 30 animals (13%). In the present study, the results obtained from CS were compared to those obtained from blood, a less invasive sample potentially useful for mass screening diagnosis of canine leishmaniasis. The positivity obtained with CS was significantly higher than those obtained from blood samples. The hybridization step was essential since it increased significantly the assay sensitivity and

guaranteed the specificity, avoiding that spurious DNA bands could be wrongly scored. A representative hybridization result was showed in the Figure 2.

Table 2. Number of positive samples after kDNA PCR-Hybridization

Sample	Number of positive
CS	27 (90%)
B	4 (13,6%)

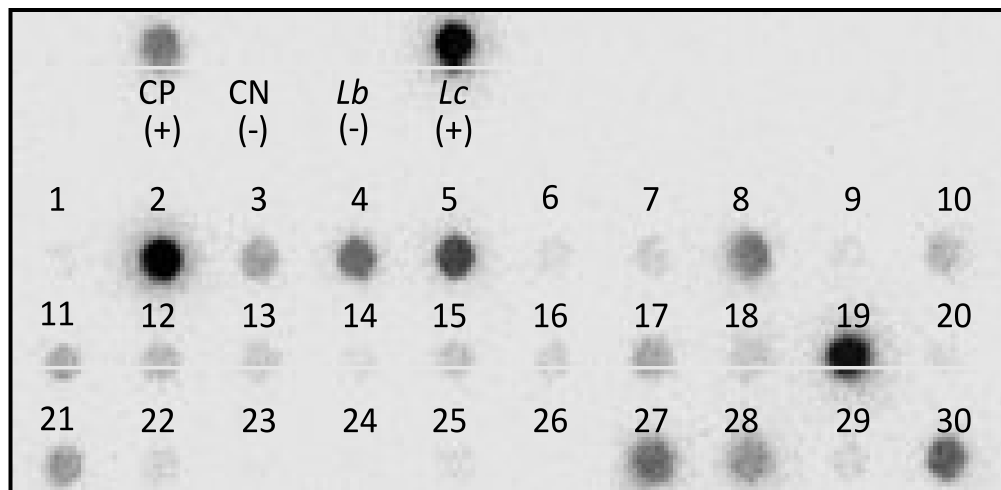

Figure 2: Representative autoradiogram of conjunctival swab PCR products obtained after hybridization with the probe labeled with $^{32}P[\alpha]dCTP$. The image was obtained using a *Bio-Imaging Analyzer* (Fujifilm). CP = dog positive sample; CN = negative control; *Lb* = *L.braziliensis* positive control; *Lc* = *L.chagasi* positive control; 1 to 30 = samples of asymptomatic dogs obtained from the right conjunctiva.

3. CONCLUSIONS

The high sensitivity obtained with asymptomatic dogs, in which the diagnosis is more difficult due the low number of parasites in the samples; allow concluding that the conjunctival swab associated to the kDNA PCR-hybridization assay provides a valuable alternative tool for the direct diagnosis of canine leishmaniasis. This approach is sensitive, non invasive, practical to perform and can be very useful for the follow-up and regular screening of dogs.

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