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BRIEF COMMUNICATION

SINGLE STEP POLYMERASE CHAIN REACTION (PCR) FOR THE DIAGNOSIS OF THE *Leishmania (Viannia)* SUBGENUS

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SUMMARY

In Brazil, the main etiologic agent of Leishmaniasis that frequently presents with mucosal involvement belongs to the *Viannia* subgenus. The therapeutic conduct in this disease depends on the parasitological diagnosis, and classical methods are restricted in identifying the agent. In this paper we describe a polymerase chain reaction (PCR), which uses primers designed from mini-exons repetitive sequences. The PCR amplifies a 177bp fragment that can distinguish *(Viannia)* from *(Leishmania)* subgenus. This test could be a useful diagnostic tool.

KEYWORDS: Leishmaniasis *(Viannia);* PCR; Identification.

INTRODUCTION

Leishmaniasis is the most diverse and complex of all vector borne diseases in terms of both its ecology and epidemiology. Despite the fact that only 30 countries have notified the disease, it is endemic in 88 countries, where a total of 12 million people are infected (WHO, 1998). Ninety percent of all cases of cutaneous leishmaniasis (CL) occur in Iran, Saudi Arabia, Syria, Afghanistan (Old World), Peru and Brazil (South America). In the last 20 years, CL has been increasing in Latin America, particularly in Brazil (DEANE & GRIMALDI Jr., 1985).

In Brazil, six species of *Leishmania* belonging to *(Leishmania)* and *(Viannia)* subgenera are responsible for CL clinical forms in human beings. *Viannia* subgenus comprises the following species: *Leishmania (V.) braziliensis*, *Leishmania (V.) guyanensis, Leishmania (V.) naiffi, Leishmania (V.) shawi* and *Leishmania (V.) lainsoni*. All these species are present in Brazil, most of them in the Amazon region (GONTIJO & CARVALHO, 2003).

Determining the infecting species is an important step in analyzing epidemiological conditions of transmission and in determining prognosis and adequate chemotherapy. Classical methods, such as microscopic analysis and isolation of the parasites in culture, are laborious and inaccurate as many species are often morphologically indistinguishable (MAGILL, 2000).

Molecular approaches have recently allowed diagnosis based on PCRs. These are usually performed with repeated gene sequences such as mini-exon, kDNA or ribosomal genes (ARANSAY *et al.*, 2000; MICHALSKY *et al*., 2002; MIRANDA *et al*., 2002). The nuclear mini-exon genes consist of 200 copies *in tandem* separated in transcribed and non-transcribed genes. The transcribed region consists of a highly conserved exon with 39 nucleotides and a moderately conserved intron among species of the same genera or subgenera. The non-transcribed region consists of a variable intergenic region among *Leishmania* species that is absent in vertebrate hosts and vectors (FERNANDES *et al*., 1994). We designed a new set of primers based on nuclear mini-exon genes and sought amplification based on subgenus *Viannia* in order to distinguish these subgenera from others and to improve the diagnosis of Leishmaniasis.

MATERIAL AND METHODS

Parasites and positive tissue samples: Strains of *(Viannia)* subgenus: *Leishmania (Viannia) braziliensis* MHOM/BR/1975/M2903 and *Leishmania (Viannia) guyanensis* MHOM/BR/1975/M4147. Strains of *Leishmania* subgenus: *Leishmania (Leishmania) amazonensis* IFLA/BR/67/PH8 and *Leishmania (Leishmania) chagasi* MHOM/BR/1974/PP75(M2682). All were kept in liquid nitrogen or cultivated in supplemented M199 medium, and positive *Leishmania* human tissue (LW) obtained from the laboratory of Prof. Lucile Floeter Winter, ICB, University of São Paulo, were used.

Reliability of the clinical samples: In order to confirm the

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sensitivity and specificity of mini-exon primers, a blind test was carried out with 92 samples from Espírito Santo. Seventy-one samples were confirmed as cutaneous Leishmaniasis in clinical, parasitological and epidemiological diagnoses and were stored in formol (LF) or embedded in paraffin (LP). 34 samples were stored in formol and 58 were embedded in paraffin. Of those stored in formol, 21 were related to other dermatological diseases.

DNA extraction: SAMBROOK *et al.* (1989) phenol/chloroform method or QUIamp (QIAGEN Co.) Kit were used for DNA extraction.

Primer design and PCR assay: Repetitive mini-exon sequences described by DEGRAVE, W. (accession number X69442) were used to design the specific primers for *L. (V.) braziliensis*. Consensus sequences for each primer were constructed manually and confirmed by DNASTAR-Laser Gene program.

The following primers which amplify a 177bp product were used: Forward (LV1) 5' CGTCTTCCGGCAACATTT 3' and Reverse (LV2) 5' CGTTAGTTGGAAGCCCAAGG 3'. A 25 µl reaction mixture containing DNA, 1 µM of each primer of 0.2 mM of dNTPs, 2.5% formamide, 1x Taq buffer (Biotools B&M Labs., S.A.) and 0.5 units of Taq DNA polymerase was placed in a thermal cycler (Eppendorf Mastercycler gradient serial No. 5331) at 95 °C for five minutes for initial denaturation, followed by 35 cycles at 95 °C for one minute, 55 °C for 30 seconds, 73 °C for one minute and then 72 °C for six minutes for final extension. Products (5 µl each) were electrophoresed on 6% polyacrilamide gel and 1.5% of agarose gel.

Cloning and sequencing - PCR products: PCR products were ligated into pGEM T-Easy vector (Promega), transformed into $CaCl_2$ competent *E. coli* DH5-α bacteria and plated on LB-ampicillin plates. White colonies were grown overnight on 3 ml LB-amp and plasmids were prepared for sequencing following the procedure for the GFX Micro Plasmid Prep Kit (Amersham/Pharmacia). A minimum of two clones were labeled by Cy5 Autoread Sequencing Kit and sequenced by ALFexpress DNA Sequencer (Pharmacia). Sequences were submitted to Genebank and the results were analyzed.

Sensitivity and specificity assays: The estimated DNA concentration was obtained by comparison of the band from genomic purified *Leishmania* DNA loaded in a 2% agarose gel with the Low DNA Mass Ladder (Invitrogen), in accordance with the manufacturer's instructions. In order to determine the PCR sensitivity, the reaction was carried out with several DNA concentrations diluted from 18pg to 0.15pg.

The specificity test was analyzed using purified human DNA (from peripheral blood mononuclear cells) and genomic DNAs from *Lu. longipalpis* and *Plasmodium falciparum.* DNAs from *Kinetoplastida* genera, *T.cruzi* (blood forms) and *Crithidia* spp (cultured organisms) were also tested.

RESULTS AND DISCUSSION

The correct identification of *Leishmania* species is crucial, as this will affect the type of clinical survey required. Control of Leishmaniasis requires knowledge of the ecology and the epidemiology of the disease.

The mini-exon genes in *Kinetoplastid* protozoa are present in 100-200 tandemly repeated copies in the nuclear genome (FERNANDES *et al*., 1994). This characteristic has been shown to be a useful tool for identifying the subgenus or *Leishmania* species. Based on these sequences, which were deposited in Genebank (number X69442), a pair of primers (LV1 and LV2) to identify the *(Viannia)* complex was designed.

In order to standardize the annealing temperature, a gradient of 12 temperatures varying from 45 °C to 65 °C was used. Clear fragments were visualized with annealing temperatures of 55 °C and 58 °C. We chose 55 °C as the annealing temperature in our PCR reaction.

The species specific primer was tested, using DNA extracted from the M2903 and M4147 strains of *L. (V.) braziliensis* and *L. (V.) guyanensis* respectively and human positive *Viannia* samples embedded in paraffin and formol (LW, LP and LF) as a positive control. As shown in Figures 1, 2, and 3 a 177bp fragment was amplified. These fragments were cloned, sequenced and *(Viannia)* subgenus was confirmed by Genebank analysis. The specificity assay was carried out and LV1 and LV2 primers did not amplify DNA from *L. (L.) amazonesis* and *L. (L.) chagasi* or other microorganisms such as *P. falciparum, T. cruzi, Crithidia* sp, human DNA and *Phlebotomus* sp. DNA *(Lu. longipalpis)*

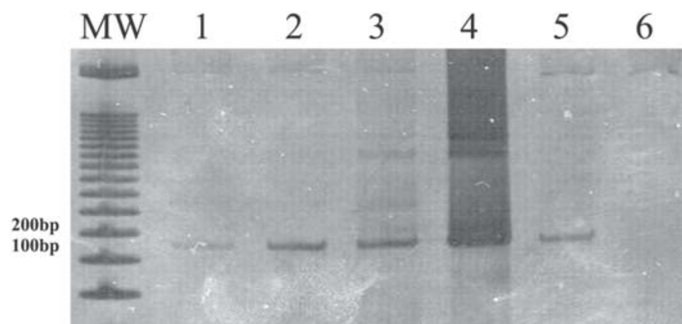

Fig. 1 - PCR amplification products. MW 50bp DNA ladder size marker (Invitrogen); 1- LFa; 2- LFb; 3- LFc; 4- *L. braziliensis* (M 2903 strain); 5- LW; 6- negative control.

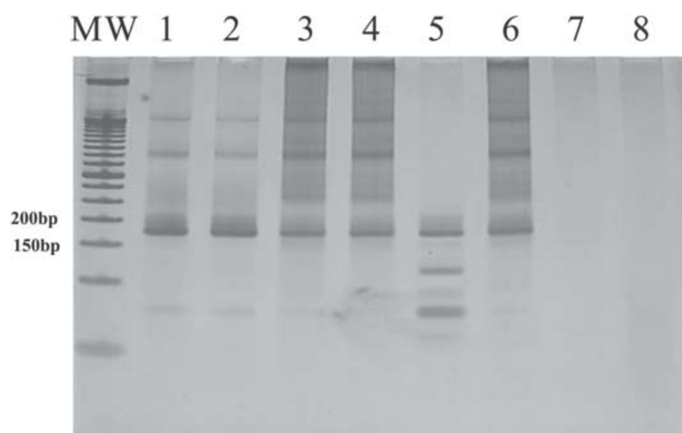

Fig. 2 - PCR amplification products. MW 50bp ladder DNA size marker (Promega); 1- LPa; 2- LPb; 3- LPc; 4- LPd; 5- LPe; 6- *L. braziliensis* (M2903 strain); 7- *L. amazonensis* (IFLA/BR/67/PH8 strain); 8- *L. chagasi* (M2682 strain)

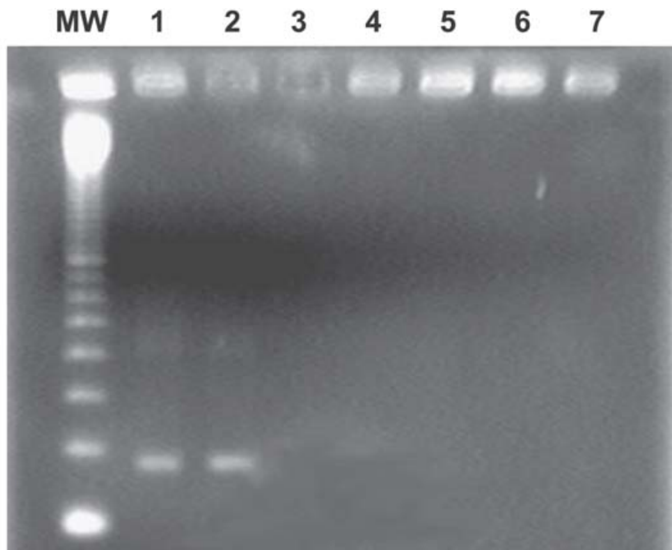

Fig. 3 - PCR amplification products. 1.5% agarose gel. Lanes MW 100pb DNA ladder size maker; 1- *L. (V.) braziliensis* (M2903); 2- *L. (V.) guyanensis* (M4147); 3- *P. falciparum*; 4- *T. cruzi*; 5- *Crithidia* sp; 6- human DNA; 7- *Lu. longipalpis*.

(Fig. 3). In the blind test using LV1 and LV2 primers, 62.1% (36/58) and 46.1% (6/13) of LP and LF respectively were PCR positive. Of the negative samples, all PCRs but one were negative.

PCR reaction detected as little as 0.15pg of *Leishmania* DNA, but this detection varied according to the DNA sample. Our reaction was able to detect 1.5 parasites, as MYLER & STUART (2000) reported that one *Leishmania* contains 100fg of DNA.

Several authors reported molecular approaches for identifying the subgenus and species of *Leishmania* (CASTILHO *et al*., 2003; RODRIGUES *et al*., 2002; UEZAT *et al*., 2001; FERNANDES *et al*., 1999) but some of these methods are either laborious or the DNA sequences used as target are usually present as single or low copy. Based on these reports, a comparison was undertaken between LV1 and LV2 mini-exon PCR and PCR-RFLP (VOLPINI *et al*., 2004). Leishmaniasis LP samples were tested for PCR-RFLP and 44% (25/58) were found to be positive for *(Viannia)* subgenus (PAIVA *et al*., 2003). These results are similar to those from LV1 and LV2 mini-exon primers. PCR-RFLP, however, needs a two-step PCR reaction to identify the *Leishmania* subgenus whereas the LV1 and LV2 mini-exon primers can identify *(Viannia)* subgenus parasites in only one step.

Based on these results, we believe that LV1 and LV2 primers could be useful in identifying *(Viannia)* complex. Studies using laboratory-infected sandflies are being carried out. Once the laboratory reaction has been standardized our next goal is to use these primers to detect infected sandflies in the field.

RESUMO

Reação de polimerização em cadeia (PCR) em etapa única para diagnóstico de *Leishmania* do subgênero *(Viannia)*

No Brasil, o principal agente etiológico da leishmaniose, apresentando freqüentemente comprometimento das mucosas, pertence ao subgênero *(Viannia)*. A conduta terapêutica no tratamento da leishmaniose depende de seu diagnóstico parasitológico e os métodos clássicos restringem sua identificação. Neste trabalho, descrevemos uma reação de PCR, utilizando primers desenhados a partir de seqüências repetitivas de mini-exons, que amplificam um fragmento de 177pb e que são capazes de distinguir o subgênero *(Viannia)* do subgênero *(Leishmania)*, tornando-se uma ferramenta útil no diagnóstico desta doença.

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