Communication/Comunicação

Comparison among three polymerase chain reaction assays on detection of DNA from *Leishmania* in biological samples from patients with American cutaneous leishmaniasis

Comparação entre três ensaios de reação em cadeia da polimerase na detecção de DNA de *Leishmania*, em amostras biológicas de pacientes com leishmaniose tegumentar americana

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ABSTRACT

Introduction: The study analyzed positivity of polymerase chain reaction (PCR) on detection of DNA from *Leishmania* in patients' samples. **Methods:** Extracted DNA was submitted to L150/L152, 13Y/13Z, and seminested PCR (snPCR). **Results:** Results were evidenced by bands of approximately 120, 720, and 670 bp for L150/L152, 13Y/13Z, and snPCR, respectively. L150/L152, 13Y/13Z, and snPCR positivity indexes were 76.9, 56.4, and 69.2 ($p>0.05$), respectively, for suspected and 93.7, 68.7, and 84.4 ($p<0.05$), respectively, for confirmed. **Conclusions:** Preliminary results showed that these assays, mainly L150/L152 and snPCR, can detect *Leishmania* DNA and carry potential on laboratory diagnosis of leishmaniasis.

Keywords: *Leishmania*. PCR. Diagnosis.

RESUMO

Introdução: Analisou-se a positividade da reação em cadeia da polimerase (PCR) na detecção de DNA de *Leishmania* em pacientes. **Métodos:** DNA extraído foi submetido a L150/L152, 13Y/13Z e PCR *seminested* (snPCR). **Resultados:** Resultados foram evidenciados por bandas de aproximadamente 120; 720 e 670pb para L150/L152, 13Y/13Z e snPCR, respectivamente. Positividades para L150/L152, 13Y/13Z e snPCR foram 76,9; 56,4 e 69,2 ($p > 0,05$), para suspeitos; e 93,7; 68,7 e 84,4 ($p < 0,05$) para confirmados, respectivamente. **Conclusões:** Resultados preliminares mostraram que os ensaios, principalmente L150/L152 e snPCR, podem detectar DNA de *Leishmania* e têm potencial para diagnóstico laboratorial das leishmanioses.

Palavras-chaves: *Leishmania*. PCR. Diagnóstico.

Leishmaniases are caused by an intracellular protozoan of the genus *Leishmania*, whose promastigote forms can be transmitted to humans by the bite of *Lutzomyia* female insect, also known as sandfly[1].

Leishmania infection occurs in most Brazilian states, and some authors believe that its dispersal is associated to anthroponotic action. The traditional clinical manifestations for American cutaneous leishmaniasis (ACL) are single or multiple cutaneous lesions, but there are asymptomatic cases as well[1].

The laboratory techniques of diagnosis involve methods of detection of the parasite, such as direct search, isolation in culture, animal inoculation, and histopathology. However, these methods present hurdles related to sensitivity[1]. On the other hand, immunodiagnostic techniques, such as the Montenegro skin test (MST), can increase the speed of the diagnosis, but they are unable to distinguish among active, inactive, or past infection[2]. Historically, a positive MST is an indicator of previous contact with the parasite through natural inoculation. MST is the most widely used complementary test for the presumptive diagnosis of *Leishmania* infection. Positivity indexes of 84% and 100% have been estimated in cutaneous and mucocutaneous forms, respectively. Moreover, MST is negative in cutaneous diffuse form and in immunocompromised patients[2].

In this context, the use of PCR-based techniques has been shown as a new option of diagnosis, mainly due to their speed and high sensitivity[1,3]. Moreover, these techniques have been developed to amplify mini-exon genes[4], DNA coding regions for subunits of the ribosomal RNA[5], and kinetoplast DNA-kDNA[6] besides other sequences of nuclear DNA[7] in clinical samples for the diagnosis of leishmaniasis. However, a major concern in the development and implementation of PCR for *Leishmania* diagnosis is the lack of standardization; many reports have been published, but very few studies have compared the different protocols[5].

One of the main targets of these protocols is the kDNA that contains minicircles, which usually have the size of 1kb and occur up to 10,000 copies per parasite[8]. The minicircle kDNA has a denominated region conserved, with approximately 100–150 bp, and a variable one, with approximately 700-1,000bp. Both regions are targets for primers designed to the PCR[8,9].

In this work, primers directed to the conserved region — L150/L152[9] — and to the variable region — 13Y/13Z[8] — were used in the PCR. In seminested PCR (snPCR), primers LINR4/LIN17/LIN19 were used to target the variable region, although their alignment was with the conserved region[6]. The positivity of those methods was assessed based on the analysis of the materials obtained from patients suspected for ACL.

For this purpose, DNA was obtained from 39 samples (biopsies and imprints on slides) of patients with suggestive lesions for ACL.

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The patients were from the regions of Southern, Southwestern (SSMG), and Rio Doce Valley (RDV), State of Minas Gerais. Among those patients, 32 (82%) out of 39 were confirmed positive for ACL by parasitological diagnosis and/or MST. Parasitological diagnosis was done under microscopic analysis of imprints obtained from outer edges of the lesions.

All procedures involving human samples were approved under register number 141/2006 by the Ethics on Human Research Committee from *Universidade Federal de Alfenas*.

DNA extraction was carried out using proteinase K digestion of skin biopsies from lesions[9]. In this methodology, DNA extraction from samples was performed with 100µL buffer solution (10 mmol/L Tris-HCl and 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0) and 100µg/mL proteinase K (final concentration), incubated at 56ºC for 3h, and homogenized from time to time. Digestion was stopped by proteinase K inactivation, by boiling it for 15 min. The samples were centrifuged, and the supernatant was used as the *Leishmania* template DNA source for the PCR reaction. For slides, the methodology used was extraction based on heating[10]. In this methodology, an area covering two imprints, previously analyzed on microscope, was scraped from each slide with a toothpick, resuspended in 20µL of double-distilled water, and transferred to 0.5mL microtubes. The samples were heated at 70ºC for 10 min and, then, centrifuged at 12,000 g for 5 min at room temperature; after which, the supernatant was used as a DNA template.

DNA obtained from the digestion of biopsies as well as the imprints was submitted to the following three PCR techniques. Amplification was carried out in a Perkin-Elmer GeneAmp® PCR System 9,700 thermocycler under different conditions for L150/L152[9], 13Y/13Z[8], and snPCR[6].

For each reaction, a negative control tube containing no template DNA was included. The positive control was also performed consisting of 10 pg of DNA extracted from axenic cultures of *Leishmania (Viannia) braziliensis* MHOM/BR/1975/M2903 and *Leishmania (Leishmania) amazonensis* MHOM/BR/1972/M2269. Amplified products were analyzed in a 1.5% agarose gel, which was stained with ethidium bromide and under 3 V/cm.

Comparative analysis among the positivity indexes obtained by the three PCR techniques was carried out using the Chi-square test, with the level of significance set at $p<0.05$. The analysis was carried out using the Epi Info™ 6.04d (CDC) software[9].

Results were evidenced by a band of approximately 120, 720, and 670 bp for L150/L152, 13Y/13Z, and snPCR, respectively (**Figure 1**). The positivity indexes obtained by the three techniques can be seen on **Table 1**. For ACL suspected cases, a larger percentage of positive results was detected in the L150/L152 PCR (76.9%) in relation to others assays, although without significant difference ($p>0.05$), which was probably due the small number of cases. However, for confirmed cases, in parasitological tests and/or MST, these results were significantly different ($p<0.05$).

The performance of L150/L152 in the present work confirmed the results obtained from previous studies[9]. However, it was noted that the snPCR technique has the possibility of detecting and amplifying larger sizes of DNA fragments with similar positivity obtained by L150/L152. Larger DNA targets present lower efficiency of amplification[11]; however, the opposite was observed in the positivity index of snPCR from ACL suspected and confirmed patients.

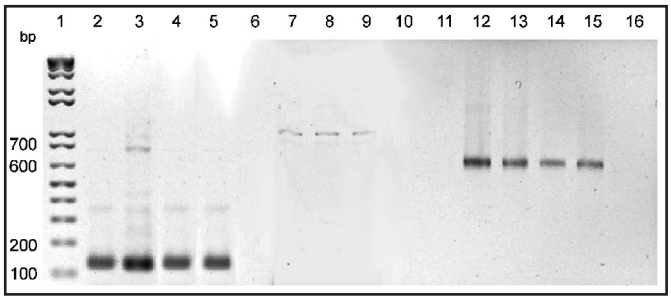

FIGURE 1 - Representative 1.5% agarose gel showing amplification products of 120 (lanes 2-6, conserved region with L150/L152 primers), approximately 720 (lanes 7-11, variable region with 13Y/13Z primers), and approximately 670 (lanes 12-16, variable region with PCR Seminested) bp from kDNA minicircles of *Leishmania* in promastigote cultures and skin biopsies from american cutaneous leishmaniasis patients.

Lane 1: Molecular size marker of 100 bp ladder**; Lanes 2, 7, and 12:** DNA from *Leishmania (Viannia) braziliensis* MHOM/BR/1975/M2903**; Lanes 3, 8, and 13:** DNA from *Leishmania (Leishmania) amazonensis* MHOM/BR/1972/M2269**; Lane 4-5, 9-10, and 14-15:** DNA of skin biopsy samples from ACL patients**; Lanes 6, 11, and 16:** negative controls**.**

TABLE 1 - Indices of positivity obtained for the PCR-based techniques applied on biological samples from ACL suspected and confirmed patients.

	Positivity index PCR (%)*			
	ACL suspected (n=39)		PAR and/or MST+ (n=32)	
PCR technique	n	%	n	%
13Y/13Z	22	56.4[a]	22	68.7[a]
L150/L152	30	76.9[a]	30	93.7[b]
snPCR	27	69.2[a]	27	84.4[a, b]

PCR: polymerase chain reaction, **snPCR**: seminested PCR, **ACL**: American cutaneous leishmaniasis, **PAR**: parasitological, **MST+**: Montenegro skin test, *Chi-square test (significant at $p<0.05$); [a, b]: equal letters mean positivity indexes that are statistically similar.

It was proposed that snPCR test is very sensitive on detection of *Leishmania*, partly because there can be about 10,000 minicircles per kinetoplast and because its sequences are known for most *Leishmania* species[3]. Results obtained by Nested PCR using other targets as ITS-1 showed a lower positivity when compared with snPCR using the kDNA[5].

Trypanosoma cruzi DNA was tested by L150/L152, 13Y/13Z, and snPCR to verify possible cross-reaction, but specific fragments were not observed (data not shown). In addition, results of PCR obtained for *Leishmania* using 13Y/13Z primers[8] as well as snPCR[3,6] did not show cross-reactivity with other trypanosomatids. Cross-reaction and low specificity for L150/L152[12] were observed. Furthermore, to minimize risks of contamination, improvements were done in snPCR in relation to the conventional Nested technique[13].

Moreover, it was proposed that the control of leishmaniasis in areas of endemicity requires a thorough knowledge of *Leishmania* ecology and epidemiology and a sensible method for the detection of the parasite, which can be capable of processing a large number of samples synchronously[6]. In this context, results obtained in three PCR assays show that these techniques can be useful for this purpose. Additionally, these methodologies have been shown capable of detecting kDNA from *Leishmania* in imprints on slides, where the shortage of material is not a limiting factor[10].

In conclusion, the preliminary results showed that three PCR assays, mainly L150/L152 and snPCR, can detect *Leishmania* DNA, including those in biological samples from ACL patients, and

that they also carry potential application on laboratory diagnosis of leishmaniasis. However, it is important to evaluate a larger amount of samples to verify the trend of L150/L152 and snPCR on producing results with greater positivity and significance, in relation to 13Y/13Z. Other studies are in progress to test the ability of the association of restriction fragment length polymorphism (RFLP) and snPCR on distinction of the different *Leishmania* species and to identify *Leishmania* DNA in other populations like dogs with visceral leishmaniasis.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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