

## Detection of *Leishmania (Viannia) braziliensis* by PCR in samples collected by scraping the lesion edges from patients of an endemic area

## Detecção de *Leishmania (Viannia) braziliensis* por PCR em amostras coletadas por raspagem de bordas de lesões de pacientes de uma área endêmica

RIALA6/1678

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Recebido: 30.06.2015 - Aceito para publicação: 22.12.2015

### ABSTRACT

American cutaneous leishmaniasis (ACL) is an infectious disease caused by *Leishmania*. Their diagnosis is performed in samples collected from the lesion biopsies, which has to be performed by physicians. For simplifying the sample collection, this study proposes a minimally invasive procedure, by scraping the lesion edges. Laboratory diagnosis by PCR was performed and compared with the microscopic examination, by analyzing 28 samples collected from patients with suspicion of ACL. Sample collected from the lesion edge with a sterile toothpick was divided into two aliquots. One aliquot was analyzed under direct microscopy, and the second by PCR, by using two primer pairs (one for *Leishmania* genus and other for *L. (V.) braziliensis*). Of 28 samples, 27 (96.43 %) showed concordant results in both methodologies (eight positive and 20 negative). The PCR methodology is an invaluable tool: (i) to determine the *Leishmania* species; (ii) to provide an alternative procedure of sample collection, when an authorized professional is not available in the respective health service; and (iii) to propose a minimally invasive procedure for collecting biological material.

**Keywords.** American cutaneous leishmaniasis, *Leishmania (Viannia) braziliensis*, PCR.

### RESUMO

Leishmaniose tegumentar americana (LTA) é uma doença infecciosa causada por *Leishmania*. O diagnóstico é realizado em material de biópsias das lesões, cuja coleta é feita por médicos. Para simplificar a coleta de amostra, este estudo propõe um procedimento minimamente invasivo, realizando-se a raspagem das bordas da lesão. O diagnóstico por PCR foi comparado com o exame microscópico, analisando-se 28 amostras coletadas de pacientes com suspeita de LTA. Cada amostra, coletada da borda da lesão com um palito estéril, foi dividida em duas alíquotas. Uma foi analisada pelo exame microscópico direto e a outra pela técnica de PCR, utilizando-se dois pares de oligonucleotídeos (um específico para gênero *Leishmania*, e outro para *L. (V.) braziliensis*). Das 28 amostras, 27 (96,43 %) apresentaram resultados concordantes em ambas as metodologias (oito positivas e 20 negativas). A PCR em material de raspado da borda de lesões foi mais sensível quando comparado com o exame direto. A metodologia de PCR apresenta vantagens para: (i) determinar as espécies de *Leishmania*; (ii) oferecer um meio alternativo de coleta de amostras, quando os serviços de saúde não têm o profissional autorizado para coletar o material de biópsia; (iii) propor um procedimento minimamente invasivo de coleta de amostra biológica.

**Palavras-chave.** Leishmaniose cutânea americana, *Leishmania (Viannia) braziliensis*, PCR.

## INTRODUCTION

American cutaneous leishmaniasis (ACL) is an infectious disease caused by protozoan of the genus *Leishmania* that affects skin and mucosa. According to World Health Organization (WHO) 1- 1.5 million new human cases of cutaneous forms occur yearly. From 2001 to 2011 around 270,500 cases were reported, with an average of 27,500 new cases/year<sup>1,2</sup>. Around 3 to 5 % patients who develop cutaneous lesions can also develop mucosal leishmaniasis<sup>1-3</sup>. In the Sao Paulo State, the incidence is approximately 400 new cases/year<sup>4</sup>. Another considerable problem is the urbanization of the infection. Autochthonous cases have been described in urban areas<sup>5</sup>. The incidence of peri-urban and urban cases has been increasing. Approximately 10 % of the population living in endemic areas is at risk for acquiring the infection<sup>5,6</sup>. ACL was also considered one of the most common dermatological syndromes diagnosed in travelers (or tourists) who had visited endemic areas<sup>6</sup>.

Species such as *L. (V.) braziliensis*, *L. (V.) guyanensis* and *L. (L.) amazonensis* can cause ACL<sup>3,6</sup>. Such parasite diversity causes different forms and manifestations, mainly in the skin and mucous membranes and in the host immune system<sup>6</sup>.

The traditional diagnosis of ACL is performed using clinical, epidemiological and laboratory methodologies. Considering the laboratory techniques, biopsies from lesions are normally processed for direct examination, after Giemsa staining, "in vitro" culture, including histopathological techniques, and more recently, molecular methods as polymerase chain reaction (PCR)<sup>7</sup>. Studies related the determination of the infecting species can cooperate with the epidemiological and preventive strategies. Our group previously evaluated a PCR for detecting *L. (V.) braziliensis* in clinical samples, which has been shown to be highly sensitive and specific<sup>7,8</sup>.

The human biopsy collection must be performed only by physicians; in addition,

it is a complex and expensive procedure<sup>1,3,4</sup>. Based on this, and in order to simplify the sample collection for molecular ACL diagnosis, the present study aimed to propose a minimally invasive procedure, simple and fast sample collection procedure using molecular and parasitological methods.

## MATERIAL AND METHODS

### Patients and samples

This study was conducted analyzing clinical samples from 28 patients with cutaneous lesions living and attending public dermatology clinics of Sorocaba Region, Sao Paulo State. All patients analyzed in this study also lived in these cities and were selected considering epidemiological risk factors for cutaneous leishmaniasis, such as close proximity to other infected patients as well as signs or symptoms of the disease. In the dermatology clinics, a complete dermatological examination was performed, and all patients presented cutaneous lesions suggestive of leishmaniasis. None of them had acquired the infection before or had been treated with drugs for leishmaniasis. Lesions were initially cleaned with antiseptics. The borders of the lesions were scraped or smears of material were obtained with the aid of a sterile toothpick. One part of samples was immediately added to a tube containing 1-2 mL of a sterile 0.85 % NaCl for DNA extraction. The other part of sample was distended directly on two glass slides for parasitological test (microscopic examination).

For negative control in PCR, DNA was extracted from three biopsies with other diseases and for positive control, from the standard WHO strain, *L. (V.) braziliensis* (MHOM/BR/1975/M2903). Promastigotes were maintained by serial passages and grown at 24 °C in 199 medium supplemented with 10 % calf serum and 0.25 % hemin 25. In the log curve phase,  $1 \times 10^8$  promastigotes were harvested, and washed twice in phosphate-buffered saline (pH 7.2) at 1,000 g for 10 min. The parasite pellets were used for DNA extraction.

## Ethical considerations

This study was performed according to recommendations of the Human Ethics Committee (CONEP-IAL, number 424.827) that approved this study.

## Microscopic method

Smears on glass slides were fixated with methanol and stained with Giemsa according to the WHO<sup>1,2</sup>. The presence of amastigotes was observed microscopically with an immersion objective (magnification 1,000X).

## Molecular diagnosis

### DNA extraction

Clinical samples and *Leishmania* WHO reference strain were crushed and digested, until complete tissue lysis, in a lysis buffer (This-HCl, 10 mM, pH 8.0; EDTA 10mM; SDS, 0.5 %; N-laurilsarcozil, 0.01 %; proteinase K, 100 µg/mL) by incubation in water bath at 56 °C. Next, DNA molecules were extracted by QIAamp® DNA Mini Kit (Qiagen), according to the manufacturer's instructions. DNA concentrations and purity were determined by the NanoDrop ND1000® (Thermo Scientific).

### PCR, targets for *Leishmania* and internal controls

*Leishmania* spp. was identified by using the primer set 150/152<sup>9</sup>, targeting a 120-base pair (bp). PCR product from a conserved region of kDNA minicircles. *L. (V.) braziliensis* complex was determined by using the primer set LU-5A/LB-3C (5'-TTTATTGGTATGCGAAACTTC-3'/5'-CGT(C/G)CCGAACCCCGTGTC-3') targeting an amplified fragment of 146-149 bp from the multicopy spliced leader RNA gene<sup>7,8,10</sup>. These reactions were run following the same conditions as described by Gomes et al<sup>7</sup> and Gomes et al<sup>8</sup>. To check PCR inhibitors, samples were assayed by using a reference gene ( $\beta$ 1- $\beta$ 2)<sup>11</sup>, in the same conditions as previously described<sup>8</sup>. The samples were tested in two replicates. Each amplification run contained two negative controls (ultra-pure water and a negative DNA sample for *Leishmania*) and one positive control (DNA extract of *L. (V.) braziliensis* promastigotes).

After thermal cycles, PCR products were electrophoresed in 2 % agarose gel and stained with ethidium bromide. DNA fragments were visualized under UV illumination. The images were analyzed by a Mini Bis Gel Imager and Documentation (DNR Bio-Imaging Systems). The size of fragments was based on comparison with a 100-bp ladder.

### Data Analysis

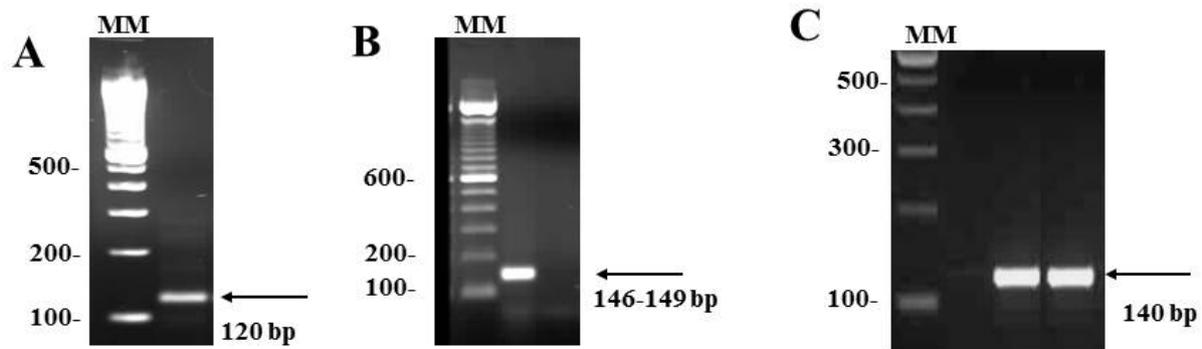
According analyses made in previous studies<sup>7,8</sup> and in the laboratory routine in our laboratory, clinical and laboratory criteria were used to establish the diagnosis. The samples were considered positive when stained tissue smears or PCR were positive. These values were used as the "consensus laboratory criteria" against which each individual diagnostic assay was compared<sup>8</sup>.

## RESULTS AND DISCUSSION

During the study-period, the 28 patients clinically suspected of having ACL referred to the public dermatology clinics were diagnosed by clinical, epidemiological and laboratorial data. Parasites were identified by, at least one of the methods employed. The central objective of this study was to investigate whether PCR or the direct microscopic examination could determine *L. (V.) braziliensis* in clinical material collected by scraping the edges of the lesions, in case physicians could not perform biopsies to collect the material.

DNA extraction and PCR inhibitors were evaluated by  $\beta$ 1- $\beta$ 2 marker that amplified a PCR fragment of human  $\beta$ -globulin gene. All DNA samples had positive amplifications showing that no substance present in DNA samples inhibited the reaction. *Leishmania* determination was made using two primer pairs: a 120 bp, specific for *Leishmania* spp, and a 146-149 bp fragment from the SL RNA gene of *L. (V.) braziliensis* complex. As an illustration, Figure 1 shows the amplified PCR products of all markers used in this study.

The overall laboratory results, showing each individual diagnostic test for clinical samples of all patients, are described in Table 1. Samples from the 28 patients presented the following results: eight



**Figure 1.** Amplified PCR products in two different target regions of *Leishmania*: 150/152, 120 bp (for *Leishmania* spp) (A) and LU-5A/LB-3C, 146 to 149 bp (for *L. (V.) braziliensis* complex) (B). B1-β2 primer pair was used as PCR control and amplified a 140 bp fragment of human β-globulin gene (C). The amplified products were resolved in 2 % agarose gels stained with ethidium bromide. Lane MM, 100 bp ladder

patients had positive results for PCR (with both primer pairs), whereas parasites were detected by parasitological methods in seven patients. No parasites were shown in the other 20 samples suggesting that patients may have other diseases.

These data suggest the possibility to use PCR in dermal scrapings, since our data showed concordance between parasitological and molecular methodologies. The collection of dermal scrapings is a methodology minimally invasive if compared with skin biopsies. Previous studies have equally described good sensitivity of PCR when compared with direct microscopy in lesion scrapings. In addition, sensitivity was similar to that of DNA samples extracted from biopsies<sup>12,13</sup>.

In conclusion, this study showed the possibility of PCR use in scraping of lesion

edges (i) to determine *Leishmania* species in positive samples (ii) to use an alternative way of collecting material, when biopsy procedures are not available at the health services, and (iii) to use a minimally invasive procedure.

## ACKNOWLEDGMENTS

This study was supported by grants from the FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil) 2011/13939-8, and the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) 303489/2012-0 for Vera L. Pereira-Chioccola.

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**Table 1.** Comparison of parasitological and molecular methods for *L. (V.) braziliensis* detection in scrapings of lesions edge from patients with cutaneous lesions

		Parasitological		
		Negative	Positive	Total
PCR	Negative	20	0	20
	Positive	01	07	08
	<b>Total</b>	<b>21</b>	<b>07</b>	<b>28</b>

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