

DNA identification of *Leishmania (Viannia) braziliensis* in human saliva from a patient with American cutaneous leishmaniasis

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Abstract: This study aims to report the amplification of the DNA of *Leishmania (V.) braziliensis*, using polymerase chain reaction, obtained from the saliva of a patient with American cutaneous leishmaniasis who did not present any lesion in the oral mucosa. Amplification produced fragments of 103 bp, an estimated size employing *Leishmania (V.) braziliensis* primers (b1 e b2). The present results revealed, for the first time, that the *in vitro* amplification of *Leishmania* DNA using samples from the salivary fluid of a patient with American cutaneous leishmaniasis is possible. However, more studies are required with a larger number of participants to evaluate the usefulness of saliva as a non-invasive sample for PCR. The development of such non-invasive technique is necessary for the diagnosis of many diseases in the future, especially infectious and parasitic ones.

Key words: cutaneous leishmaniasis, saliva, *Leishmania*, polymerase chain reaction, non-invasive diagnosis.

INTRODUCTION

Leishmaniasis, a disease that affects humans, is caused by different species of parasites that can be differentiated only by biochemical, immunological, molecular and pathological methods. Sensitivity – its capacity of detecting microorganisms – and the speed with which the identification of the parasite can be made comprise the main advantages of techniques based on DNA detection.

Advances in molecular biology have enabled the development of specific tools for diagnosing various diseases, allowing the establishment of a more precise diagnosis and prognosis (1, 2). Among the molecular methods that have been developed with high sensitivity and specificity that can be applied in taxonomy diagnosis, the polymerase chain reaction (PCR) is presented as an option in diagnosis of leishmaniasis (3-5). It has the potential of becoming an additional

diagnostic tool, allowing the establishment of a correct diagnosis and a more precise prognosis.

It has been reported in the literature that it is possible to detect the causative agent of the visceral leishmaniasis (VL) in tissue samples, blood, urine, feces and swabs of human nasal and oral secretions (6). However, there are no records in the medical literature concerning the presence of etiologic agents of American cutaneous leishmaniasis (ACL) in the oral fluids, thus, the objective of the present study was to use molecular methods such as PCR to detect *Leishmania* in these secretions. Knowing of the high sensitivity and specificity of PCR, this molecular tool was employed to verify the possibility of detecting *Leishmania* DNA in the saliva of a patient with clinical diagnosis of ACL. Because of the complex diagnosis of the disease, the detection of *Leishmania* DNA in saliva could be an option in the parasitological investigation (7, 8).

The current study presents the case of a 35 years old individual from a rural area (farmer), who in August 2007 showed a skin lesion on the left calf and the temporal region. The Montenegro skin test was positive and clinical cure was obtained with the use of N-methylglucamine, 20 mg/kg/day for 20 days. Nine months later, the patient returned with skin lesions on the face, distributed next to the left angle of the mouth, on right and left pre-auricular region, on the chin and on the right lateral neck; however, there were no lesions in the oral mucosa (Figure 1).

Besides the patient with ACL, six other patients with VL and a healthy subject (whose saliva was collected and included in the reaction) were also tested.

The oral fluid was collected during the medical examination of the patients, put in a 1.5 mL sterile Eppendorf microtube, and frozen at -20°C until the DNA extraction. The DNA extraction method employed phenol/chloroform and precipitated with ethanol (4, 9).

POLYMERASE CHAIN REACTION

Primers described in the literature were used in the PCR. The reaction was standardized by the Laboratory of Molecular Biology of the Federal University of Mato Grosso do Sul, according to Lima Júnior *et al.* (10). The DNA samples used as controls were provided by the Leishmaniasis Laboratory of the Research Center René Rachou/Fiocruz (Belo Horizonte, Brazil): *L. (L.) chagasi* (MHOM/BR/74/PP/75), *L. (L.) amazonensis* (IPLA/BR/67/PH8) and *L. (V.) braziliensis* (MHOM/BR/75/M2903), taking into account the expected product size and annealing temperatures of primers. For *L. (L.) chagasi* the primers used were RV1 (CTTTTCTGGTCCCGCGGGTAGG) and RV2 (CACCTGGCCTATTTTACACCA), with the expected product of 145 bp; for *L. (L.) amazonensis* the primers were a1 (TGCGAGGATAAAGGGAAAGAA) and a2 (TGCCCTGACTTGTCATGTCTA), with the expected product of 62 bp; and for *L.*



Figure 1. The patient showing papular lesions on pre-auricular regions and on the right cervical region, a scaly lesion on the left angle of the mouth and a pustular lesion on the chin.

(*V. braziliensis*, the primers used were b1 (GTGGGCGTATCTGCTGATGAC) and b2 (CAAAAAGCGAGGGACTGCGGA), with the expected product of 103 bp. These data are displayed in Table 1.

PCR Conditions

- With a volume of 25 µL RV1/RV2: Phoneutria 2X buffer (Phoneutria Biotecnologia e Serviços Ltda., Brazil), 0.2 mM dNTPs, 0.3 mM MgCl₂, 0.16 pmol RV1/RV2 primers, Taq polymerase 4U (Phoneutria Biotecnologia e Serviços Ltda., Brazil), 0.5 of mL DNA, 19.4 of mL water and thermal cycler BIOER XP (BIOER Techonology Co. Ltda., China). The cycle conditions were 95°C for five minutes, 40 cycles at 94°C for 30 seconds, 70°C for one minute, 72°C for one minute and final extension at 72°C during ten minutes.
- With a volume of 25 µL a1/a2: Phoneutria 2X buffer (Phoneutria Biotecnologia e Serviços Ltda., Brazil), dNTPs 0.2 mM, 0.3 mM MgCl₂, 0.4 pmol a1/a2 primers, Taq polymerase 4U (Phoneutria Biotecnologia e Serviços Ltda., Brazil), 0.5 mL of DNA, 18.8 mL of water and a thermal cycler BIOER XP (BIOER Techonology Co. Ltda., China). The cycle conditions were 95°C for five minutes, 35 cycles at 95°C for 30 seconds, 55°C for 1.5 minute, 72°C for 1.5 minute and final extension at 72°C for ten minutes.
- With a volume of 25 µL b1/b2: Phoneutria 2X buffer (Phoneutria Biotecnologia e Serviços Ltda., Brazil), 0.2 mM dNTPs, 0.3 mM MgCl₂, 1 pmol a1/a2 primers, Taq polymerase 4U (Phoneutria Biotecnologia e Serviços Ltda., Brazil), 0.5 mL of DNA, 17.3 mL of water and thermal cycler BIOER XP (BIOER Techonology Co. Ltda., China). The cycle conditions were 95°C for five minutes, 35 cycles at 95°C for 30 seconds, 70°C for 1.5 minute, 72°C for 1.5 minute and final extension at 72°C for ten minutes.
- During PCR cycles, in the denaturation step, the temperature was raised up to 95°C for five minutes. In the following steps, it was maintained at 95°C for 30 seconds and in the annealing step, it was reduced to 70°C for one minute to *L. chagasi*, 55°C for *L. amazonensis* and 70°C for *L. braziliensis*. During polymerization, the temperature was reduced to 72°C for 90 seconds for all species. Finally, PCR cycles were repeated 40 times for *L. chagasi* and 35 times for *L. amazonensis* and *L. braziliensis*.

The electrophoresis was performed on agarose gel with 2% tris-acetate-EDTA (TAE) 1X buffer, pH 8.0, 80 V and 400 mA (0.04 M Tris-acetate, EDTA 0.001). The gels were stained with ethidium bromide (0.5 mg/mL) and visualized with ultraviolet light.

When all the PCR conditions for *Leishmania* DNA amplification were established, and the primers RV1 and RV2 specific to the species *L. (L.) chagasi* and a1/a2 specific to *L. (L.) amazonensis* were tested, the results were not satisfactory, since they did not permit the amplification of DNA from the saliva of the tested patients. However, when the primers b1/b2, specific to *L. (V.) braziliensis* were employed, PCR was successful in the detection of DNA in

Table 1. Primers and DNA controls for the species *L. (L.) chagasi*, *L. (L.) amazonensis* and *L. (V.) braziliensis* used in PCR for identification of *Leishmania* DNA in the saliva of patients with ACL

Species	Primer		DNA control	Expected product
<i>L. (L.) chagasi</i>	RV1	CTTTTCTGGTCCCGCGGGTAGG	MHOM/BR/74/PP/75	145 bp
	RV2	CACCTGGCCTATTTTACACCA		
<i>L. (L.) amazonensis</i>	a1	TGCGAGGATAAAGGGAAAGAA	IPLA/BR/67/PH8	62 bp
	a2	GTGCCCTGACTTGCATGTCTA		
<i>L. (V.) braziliensis</i>	b1	GTGGGCGTATCTGCTGATGAC	MHOM/BR/75/M2903	103 bp
	b2	CAAAAAGCGAGGGACTGCGGA		

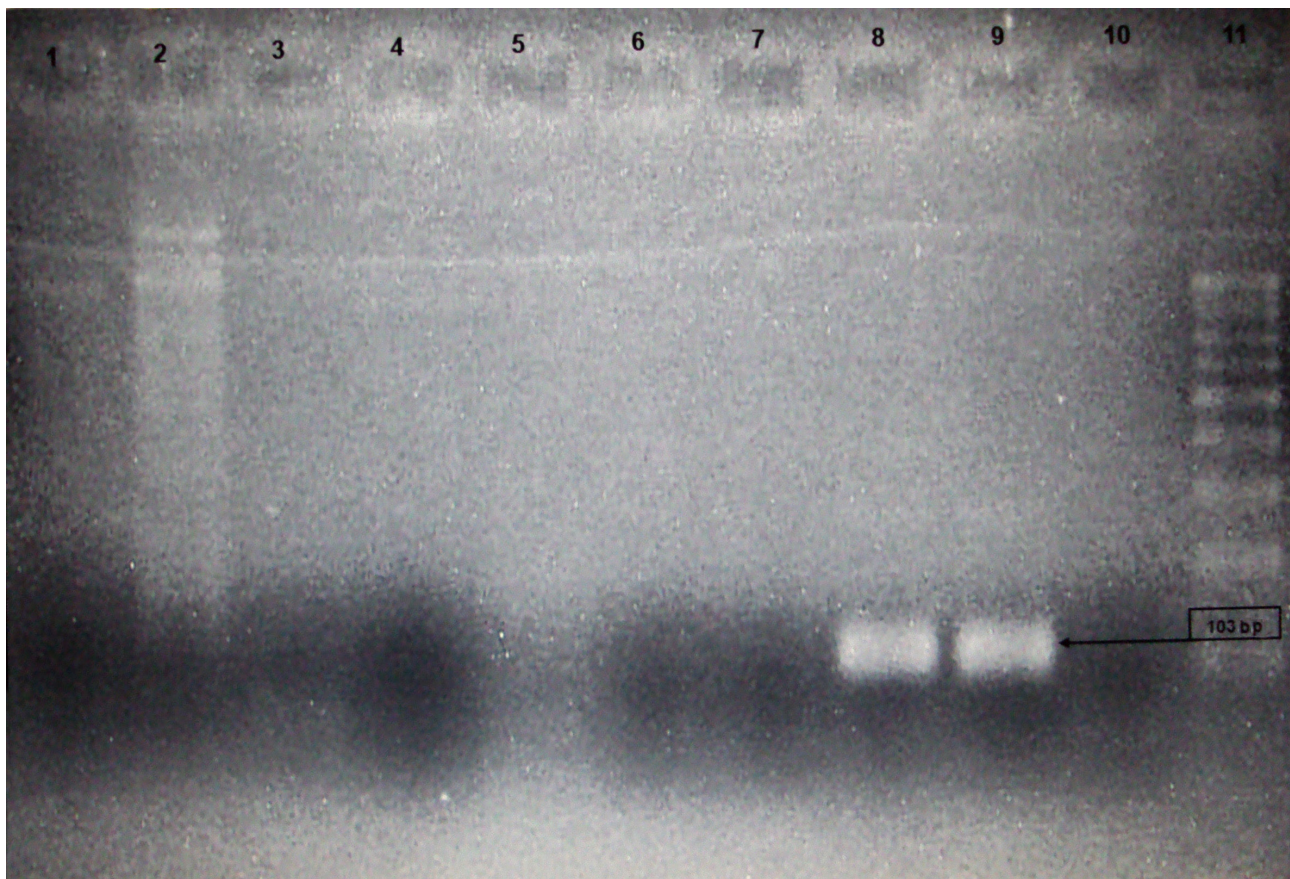


Figure 2. PCR products from samples of oral fluid showing amplification by PCR of *L. (V.) braziliensis* (lane 8) obtained from a patient with American cutaneous leishmaniasis (amplification of 103 bp). The lanes 1, 3, 4, 5, 6 and 7 identify samples of patients with visceral leishmaniasis; in lane 2, a sample of a healthy control; in lane 9, a positive control; in lane 10, a negative control; lane 11 is the marker that determines the pattern of the molecular size – 103 bp.

the saliva of the patient with ACL (Figure 2). In ACL, this species is very frequent and skin and mucosal samples subjected to PCR enables the identification of 39.2 to 93.8% of the parasite prevalence (3, 9, 11). *L. (V.) braziliensis* DNA was also detected in the salivary fluid of two more patients with mucocutaneous leishmaniasis, but these results are still under evaluation.

The PCR technology is very flexible, allowing a series of adaptations that permits its use in the analysis of a wide variety of substrates. In the literature, there are numerous studies and reports describing the use of salivary secretion as a potential biological fluid sample for laboratory tests. Its diagnosis value has been corroborated by studies using body fluids, whereby it is possible to analyze specific substrates that are important for clarifying disease diagnosis (12, 13). Oral fluids may be used for detection and characterization of microorganisms by molecular tests on the grounds of providing a

convenient source of microbial DNA (12-14). An important aspect to consider is that saliva collection is simple and noninvasive. It does not involve critical biological material due to the fact that it is dispensed directly from the mouth into the tube used for transportation. It is not complex, and decreases the possibility of contamination and technical failures since it does not depend on the intervention of technicians in this phase. Among the other advantages of using saliva as a substrate is its easy access to the mouth with a noninvasive procedure (12, 13, 15). When compared with samples of blood and urine, the transportation and preservation of salivary substrates are also simpler and cheaper (15). The present study can be considered pioneering, since it was the first to be able to detect the parasite *L. (V.) braziliensis* in the saliva sample of a patient with ACL utilizing PCR. To validate this molecular method employing saliva as a substrate in

the diagnosis of leishmaniasis, the research continues with a larger number of cases.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Committee on Research of the Federal University of Mato Grosso do Sul, process number 1152/08.

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