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Use of PCR assay to detect *Leishmania*-DNA in saliva from patients suffering American Cutaneous Leishmaniasis

Néstor Oswaldo Añez*, Gladys Elena Crisante, Agustina Rojas

Investigaciones Parasitológicas "J.F. Torrealba", Departamento de Biología, Facultad de Ciencias, Universidad de Los Andes, Mérida, 5101, Venezuela.

RESUMEN

Uso de PCR para detectar ADN de *Leishmania* en saliva de pacientes con leishmaniasis cutánea americana

Introducción: Leishmaniasis cutánea americana (LCA) afecta sobre un millón de individuos anualmente en el mundo, invadiendo piel, mucosa y fluidos incluyendo saliva. La detección de ADN de Leishmania en saliva, ha sido propuesta como método no-invasivo.

Objetivo: Evaluar el uso de ensayo de PCR-multiplex convencional en saliva, para detectar ADN de *Leishmania* en pacientes con LCA.

Materiales y Métodos: Individuos con LCA presentando lesiones activas (18) y cicatrizadas (3) fueron seleccionados para evaluar PCR en saliva para detectar ADN de *Leishmania*. Saliva de controles negativos fueron incluidas (n=3) para comparación. En todos los casos muestras de 1 mL de saliva fueron colectadas.

Resultados: La amplificación de ADN de *Leishmania* en saliva de pacientes con lesiones cicatrizadas y activas, reveló *Leishmania braziliensis* en 78% de pacientes pretratados y en 28% de pacientes durante y después del tratamiento.

Conclusión: El ensayo de PCR resultó útil para identificar *L. braziliensis* en saliva de pacientes con LCA**.**

ABSTRACT

Introduction: American cutaneous leishmaniasis (ACL) affects over one million people annually worldwide. Its spread enables *Leishmania*-parasites invading the skin, mucosa, and body-fluids including saliva. Detection of *Leishmania*-DNA in saliva, has been proposed as a non-invasive method.

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*Autor para correspondencia:
Néstor Oswaldo Añez, Investigaciones Parasitológicas "J.F. Torrealba", Departamento de Biología, Facultad de Ciencias, Universidad de Los Andes, Mérida, 5101, Venezuela.
Telephone: +58 414 7174 627.
E.mail: noar1510@gmail.com
https://revistabiomedica.mx.

Objective: To evaluate the use of multiplex conventional PCR-assay in saliva to identify *Leishmania*-DNA from patients infected with ACL.
Materials and Methods: Individuals with ACL were selected to evaluate a PCR-based saliva tool to identify *Leishmania*-DNA, 18 with active lesions and 3 with scars of previous healed ulcers, evaluated 2 years post-treatment. Saliva from 3 negative controls were included for comparison. Saliva samples (1 mL-each) were collected to be processed before, during and after treatment.
Results: Amplification of *Leishmania*-DNA in saliva from patients with healed and active lesions, revealed *Leishmania braziliensis* in 78% pre-treated and 28% during and after the treatment.
Conclusions: PCR-assay resulted useful identifying *L. braziliensis* in saliva from ACL-patients.

INTRODUCTION

American cutaneous leishmaniasis (ACL), a neglected tropical disease, is considered among the major threats to human populations living in areas where it is endemic. This form of leishmaniasis is the most common worldwide, the estimated number of cases are registered to be over 1 million annually. Its wide clinical spectrum, along with the many species of sand flies associated with *Leishmania* parasites transmission, make ACL a complex and high-risk disease, which ranges from subclinical infection to notable skin or mucosal lesions, affecting hosts' daily activity and quality of life (1-4).

The wide spread of *Leishmania* through the lymph node-netting and blood circulation, enables the parasite to invade different areas of the skin, mucosa, and the various body-fluids including urine, sperm, pharyngeal secretion, and saliva (5). Detection of *Leishmania*-DNA in leucocytes from the blood of patients with ACL, may explain subclinical case emergence, lesion recurrence, and mucosal lesion appearance. Additionally, previous reports show evidence of circulating *Leishmania*-DNA in patient blood samples years after clinically epithelized lesions or after traumatic events at different time post-therapy, suggest the long lasting of *Leishmania*-infection, as well as its capacity to evade some therapeutic protocols (6-8).

More recently, the detection of *Leishmania*-DNA in saliva, from patients infected with distinct species and in different clinical conditions, has been reported from diverse parts of the world. This includes both immunocompetent individuals without oral mucosa injuries and immunocompromised patients with HIV/AIDS infection, using in most cases, PCR techniques (9-13).

Here, we evaluated the use of a multiplex conventional PCR assay in saliva fluid to specifically identify *Leishmania*-DNA from patients infected with ACL, before, during, and after receiving specific treatment for *Leishmania*-infection.

PATIENTS AND METHODS

During the period 2015-2022, patients visited the Center for Parasitological Research at University of Los Andes, Mérida, Venezuela, for a leishmaniasis diagnosis. They were selected to carry out a study on the detection of *Leishmania*-DNA in saliva samples, using a multiplex conventional PCR assay.

The selected group consisted of 24 individuals, 18 (75%) suffering active lesions, 3 (12.5%) showing scars of previously healed or epithelized ulcers evaluated 2 years post-treatment without signs of recidivism, and 3 (12.5%) negative control patients that presented non-leishmaniasis related lesions.

As routinely done at the research center, the selected individuals were diagnosed for ACL following previously established criteria, including: **i.** the presence of active lesions; **ii.** positive leishmanin-skin test (LST) showing indurations > 5mm in diameter at 48 hours; **iii**. microscopic detection of *Leishmania*-amastigotes from samples taken at the site of lesions, which were fixed with methanol and stained with 10% Giemsa in phosphate buffer at pH 7.2, and **iv.** positive PCR assays from the ulcer samples to detect specific *Leishmania*-DNA.

To confirm the presence of *Leishmania*-DNA in samples taken from the patient's lesions, a multiplex conventional PCR assay using primers for the spliced leader RNA (mini-exon) gene sequence, was carried out. The primers and their sequences were

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as follows: **LU-5A** 5' TTT ATT GGT ATG CGA AAC TTC 3'; **LB-3C**- 5' CGT (C/G)CC GAA CCC CGT GTC 3'; **LM-3A**- 5' GCA CCG CAC CGG (A/G)CC AC 3' and **LC-3L**- 5'GCC CGC G(C/T) G TCA CCA CCA T 3', following methodology and conditions previously reported (14,15).

After verifying the ACL-case, and confirming the species of *Leishmania* causing the infection, the infected-patients were treated following weekly intralesional infiltrations with a meglumine antimoniate compound, consisting of a 1.8 mL solution made up of 90 mg/mL Sb^{5+} concentration combined with 2% lidocaine in a 1:3 proportion. The anti-leishmanial protocol, routinely used in our research center, proved to be effective enough to heal the ulcers in patients included in previous cohorts (2).

Before starting the treatment, from each of the involved individuals (infected and controls) a sample of 1mL of saliva-fluid was collected into a sterile 1.5 mL microcentrifuge tube (Eppendorf®) kept at -20°C until use. In addition, saliva's diagnostic was repeated during the treatment, and at the end of the therapy when the healing occurred.

The extraction of *Leishmania*-DNA from saliva fluid was carried out as follows: 250 µL Digisol buffer and 5 µL of [20mg/mL] proteinase K (Qiagen GmbH, Germany) were added to each saliva sample, vortexed for 30 secs, and incubated at 37°C overnight. Afterwards, 300 µL of 4M-ammonium-acetate was added to each tube and vortexed for 30 secs repeatedly for 15 min, and then centrifuged for 15 min at 980 xg at 10°C, transferring supernatant to new sterile tubes. Then 1 mL of 100% ethanol was added to the supernatant, homogenized inverting gently for DNA precipitation followed by centrifugation at 980 xg for 15min. Supernatant was then poured and pipetted off and 1 mL of 70% cold ethanol was added and the tubes, centrifuged 15 min at 980 xg, discharging the ethanol. The *Leishmania*-DNA pellet was dried in a Speed-Vac concentrator during 15 min, and then eluted in 100 µL of TE buffer and stored at -20°C until further analysis.

For identification of *Leishmania*-DNA in saliva samples, the primers, PCR-assay conditions, and electrophoresis methodology, were the same as referred above for detecting *Leishmania* in tissue samples from the patient lesions (14, 15).

ETHICAL CONSIDERATIONS

The study was conducted in accordance with the Declaration of Helsinki. A written consent including the agreement of prior information (API forms) was obtained from all the patients or their representative in case of children, in order to comply with the criteria established by the Biomedical Committee of the National Research Council of Venezuela.

RESULTS

The group of 21-*Leishmania*-infected patients, was made up of 5 (24%) females and 16 (76%) males with a ratio 0.3♀:3♂, showing an average (X±SD) age of 39±14 years (range:5-65 years), with a total of 38 lesions (1.8±1.2 lesions, range:1-6 lesions) and a time of ulcer evolution equivalent to 2.4±1.6 months (range: 0.5-8 months). Details of the patients' baseline characteristics are shown in Table 1 and in Figure 1.

The diagnostic methods used in the research, revealed positive results for detection of *Leishmania*-infection in all the selected patients. This included positive LST, presence of *Leishmania*-amastigote forms, and identification of *Leishmania braziliensis*-DNA in samples from lesions by a multiplex conventional PCR assay (Table 1).

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Table 1. Detection of *Leishmania braziliensis*-DNA in saliva from patients with ACL

Patients baseline characteristics						Diagnostic criteria for ACL				PCR assay to detect Leishmania DNA in saliva of patients with ACL		
Patient ID	**Date of diagnostic**	**Age (years)**	**Gender**	**N° of lesions**	**Time of evolution (months)**	**Clinical presentation**	**LST**	**Presence of amastigotes**	**PCR# in biopsy**	**Pretreated**	**During treatment**	**Post-treatment**
FBJFT-1*	2015	28	M	1	1	**Scar**	+**	+**	+**	ND	ND	+
LMJFT-2*	2016	40	F	1	1.5	**Scar**	+**	+**	+**	ND	ND	+
FCJFT-3*	2016	43	M	3	3	**Scar**	+**	+**	+**	ND	ND	+
CBJFT-4	2018	33	M	3	0.5	**Active ulcer**	+	+	+	+	-	-
SIJFT-5	2018	35	M	2	2	**Active ulcer**	+	+	+	+	-	+
MPJFT-6	2018	60	F	1	1	**Active ulcer**	+	+	+	+	+	+
ZBJFT-7	2018	16	M	6	2	**Active ulcer**	+	+	+	+	+	-
QFJFT-8	2018	45	M	1	2	**Active ulcer**	+	+	+	+	-	-
CFJFT-9	2018	35	M	1	3.5	**Active ulcer**	+	+	+	-	-	-
RFJFT-10	2018	41	M	2	5	**Active ulcer**	+	+	+	+	-	-
JBJFT-11	2019	52	M	1	2	**Active ulcer**	+	+	+	+	-	-
OFJFT-12	2019	37	M	1	8	**Active ulcer**	+	+	+	+	+	+
HBJFT-13	2019	60	M	1	1.5	**Active ulcer**	+	+	+	+	+	+
CSJFT-14	2019	45	M	1	1	**Active ulcer**	+	+	+	+	+	+
GPJFT-15	2020	45	M	1	3	**Active ulcer**	+	+	+	+	-	-
AMJFT-16	2020	29	F	4	3	**Active ulcer**	+	+	+	-	-	-
JAJFT-17	2020	5	M	3	3	**Active ulcer**	+	+	+	+	-	-
YNJFT-18	2021	26	M	1	2	**Active ulcer**	+	+	+	-	-	-
LMJFT-19	2022	34	M	2	2	**Active ulcer**	+	+	+	+	-	-
AFJFT-20	2022	46	F	1	2	**Active ulcer**	+	+	+	-	-	-
CDJFT-21	2022	65	F	1	2	**Active ulcer**	+	+	+	+	-	-
LPJFT-NC-1	2018	31	F	NC	None	**NA**	-	NA	NA	-	NA	NA
YQJFT-NC-2	2019	47	F	NC	None	**NA**	-	NA	NA	-	NA	NA
MMJFT-NC-3	2022	64	F	NC	None	**NA**	-	NA	NA	-	NA	NA

*: Cured cases evaluated 2 years post-treatment; **: Results detected when suffering active lesion ND: Not done; NA: Not apply; NC: Negative control; LST: Leishmanin skin test; -: Negative result; +: Positive result; #: PCR revealing *Leishmania (Viannia) braziliensis.*

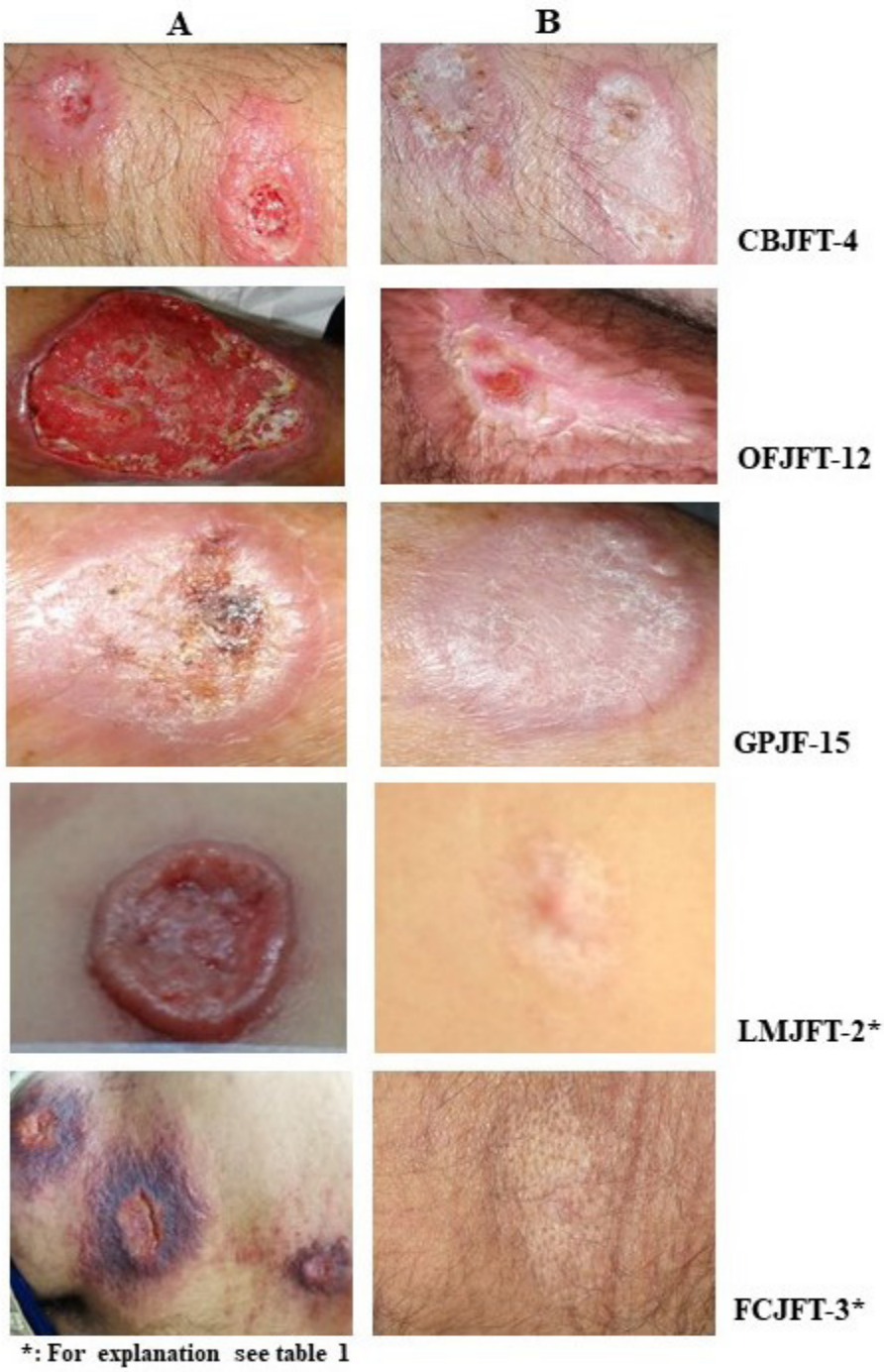

Figure 1. Active lesions (A) and post-treatment scars (B) from ACL patients with *Leishmania braziliensis*-DNA in saliva (* two years after treatment).

Regarding the amplification of *Leishmania*-DNA in saliva, taken from patients who had presented or were presenting active ACL lesions, the results revealed presence of *Leishmania (Viannia) braziliensis*. The positive samples showed bands of 149 bp, which matched with results obtained during identification of parasite causing the patients active ulcers (Figure 2). Such a similarity was observed in samples from the 3 patients who presented scars, product of previous lesions

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suffered 2 year-previously. It is also worth noting that the presence of *Leishmania*-DNA in these cases (FBJFT-1, LMJFT-2, FCJFT-3) demonstrated long-lasting persistence of the parasite in those patients who showed neither signs of cutaneous lesion-reactivation nor development of oral manifestation attributable to *Leishmania*-infection. Additionally, 14 out of 18 (78%) pre-treated patients with active ulcers, revealed *Leishmania*-DNA in saliva, an event also detected in 28% of the patients during and after the anti-*Leishmania* treatment with a pentavalent antimonial compound.

Details of the results, including active lesions, scars from previously healed lesions and the identification of *Leishmania braziliensis* in saliva samples, are shown in Table 1, and Figures 1 and 2, respectively.

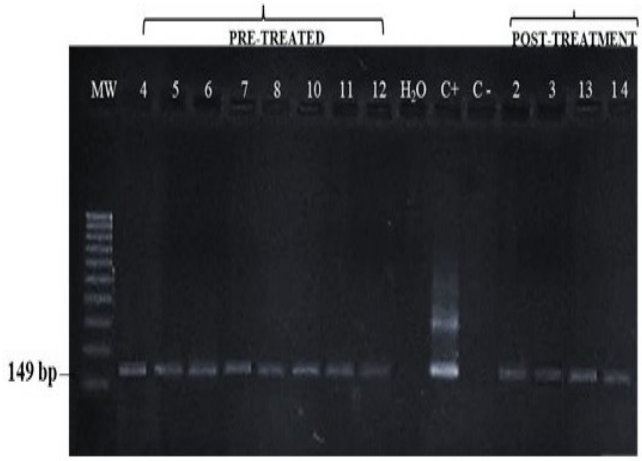

Figure 2. *Leishmania braziliensis*-DNA amplification in samples of saliva selected from patients suffering American Cutaneous Leishmaniasis. Ethidium bromide-stained gel showing PCR amplification products. Left: Lanes 4-8,10-12 samples from pre-treated patients. Right: Lanes 2-3 samples from patients taken 2 years post-treatment. Lanes 13-14 samples from patients with recently healed lesion. Lane C+: Positive control (*Leishmania*-DNA from promastigotes culture medium). Lane C-: Negative control. MW: Molecular weight marker. bp: base pairs. For patient's sample identification number see Table 1.

DISCUSSION

Here, we used a multiplex conventional PCR assay in saliva fluid to detect and specifically identify *Leishmania*-DNA before, during, and after ACL-patients receiving specific anti-leishmanial treatment. The sampling was carried out in patients unquestionably infected by *L. braziliensis* who were previously diagnosed by clinical, parasitological, immunological (LST), and molecular (PCR) methodologies.

The results demonstrated the presence of *L. braziliensis*-DNA in saliva samples from 78% of the patients with active lesions, prior to receiving specific therapy, and in 28% of the same group when evaluated during the treatment, and/or shortly after the lesion healed. In addition, similar findings were also detected in 38% of saliva samples from patients who had received complete therapy for ACL, including 3 of them with scars, two years post treatment, and 5 with recently clinically healed ulcers.

Our findings seem to indicate that saliva is a habitual destination for *Leishmania* causing ACL, after the parasite spreading from the site of the lesion. This fact finds support in previous reports involving saliva, and other fluids, as well as some structural tissues of the oral cavity in CL-patients (9, 13, 16).

The study also revealed that the presence of *L. braziliensis* in saliva, occurred irrespective of the patients' age, gender, number of lesions, and the ulcer's stage of development. These findings suggest a biological characteristic of *Leishmania*-parasite to invade saliva, and other host's fluids and tissues, after spreading over from the primary lesion. This feature seems to suggest a parasite seeking shelter from the host response, enabling a long-lasting persistence, as demonstrated in other members of the family *Trypanosomatidae* (17).

All the above appears to indicate that the presence of *Leishmania*-parasite in saliva-fluid of infected human hosts, is a long-lasting phenomenon, which may be safe, and easily detectable by using a highly sensible and specific molecular techniques such as the PCR assay. These techniques should permit

establishing an accurate diagnostic tool for ACL both for clinical study, and epidemiological surveys.

Based on the presented findings, as well as the observed accuracy, efficacy, easy usage and cost effectiveness, PCR assay in saliva fluid should be considered a routine diagnostic tool to specifically identify *Leishmania*-DNA from patients infected with ACL, extensive to other forms of leishmaniasis (i.e., VL). In addition, this methodology may help unveil subclinical or inapparent infections in afflicted populations and reveal the hidden background of this underestimated complex disease in areas where it is endemic (10, 18, 19, 20).

Finally, the potential advantage offered by the use of a PCR-based saliva diagnostic-tool as an easy, inexpensive, safe, and non-invasive approach for *Leishmania*-detection, may derive benefits for early identification, and treatment for *Leishmania* and other parasites, as well as for general medical applications or research purposes.

CONCLUSIONS

The results obtained in the research study led us to reach the following conclusions: **i.** The shown accuracy, non-invasive, safe, easy, cheap, added to its sensitivity and specificity, make the PCR-based saliva tool useful to identify *Leishmania*-DNA for clinical, and epidemiological purposes. **ii**. The evaluated multiplex conventional PCR assay revealed *L. braziliensis*-DNA in 78% saliva samples from patients bearing active lesions, previous to receive anti-*Leishmania* treatment, as well as in 28% and 38% samples from patients during, and after treatment, respectively. **iii.** The used PCR assay was also able to identify *L. braziliensis-DNA* in saliva, irrespective of the patients' age, gender, number of lesions, and the time of ulcer's evolution.

CONFLICT OF INTEREST

The authors declare they have not conflict of interest.

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