Direct Diagnosis of *Leishmania* Species on Serosity Materials Punctured From Cutaneous Leishmaniasis Patients Using PCR-RFLP

Homa Hajjaran,¹ Farzaneh Vasigheh,¹ Mehdi Mohebbali,^{1*} Sasan Rezaei,¹ Setareh Mamishi,² and Soroure Charedar¹

¹Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

²Departments of Infectious Diseases, Pediatric Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

This study was aimed at identifying the *Leishmania* species using serosity materials punctured from skin lesions of cutaneous leishmaniasis (CL) patients by using internal transcribed spacer1 (ITS1) polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). We used the PCR-RFLP on 60 parasitological confirmed CL patients who referred to leishmaniasis laboratory from the School of Public Health, Tehran University of Medical Sciences. The PCR-RFLP could correctly detect 51 *Leishmania* species of the 60

confirmed positive specimens, where all the other 10 parasitological (microscopy and culture) negative samples that were prepared from other bacterial- and fungal-infected lesions had negative results. The results also revealed that *Leishmania major* was the dominant species (53.3%). This study suggests that the PCR-RFLP assay with serosity materials punctured from CL patients using *Hae* III enzyme is useful for the rapid identification of *Leishmania* species. J. Clin. Lab. Anal. 25:20–24, 2011.

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Key words: Cutaneous leishmaniasis; PCR-RFLP; identification; human; lesion serosity material

INTRODUCTION

Leishmaniasis is a major public health problem with 1.5–2 million new cases annually and with up to 350 million people at risk in the world (1). At least 20 species and subspecies of *Leishmania* have been recorded as being infective to humans. Many of them cause extensive morbidity and are responsible for a wide spectrum of clinical signs and symptoms in the world. Correct diagnosis of the disease is very important for the selection of appropriate treatment and reduces its complications (2). As cutaneous leishmaniasis (CL) is clinically similar to other skin diseases, various techniques have been introduced to diagnose its agent in affected lesions (3). Diagnosis of CL due to its varied symptoms with different species is difficult. The classic laboratory diagnosis methods, such as examination of skin lesions by using smears, cultures and histopathological examinations, are highly specific but its sensitivity is low and variable (4,5). In addition, they are time

consuming, usually requiring experienced technicians and cannot identify the species of *Leishmania* parasite. For improving the detection and characterization of *Leishmania* spp, in the past two recent decades, a few number of polymerase chain reaction (PCR) assays with high sensitivity were used for amplifying different repeated sequences, including ribosomal (6,7), minixon and mini/maxicircle kDNA, or conserved and variable regions (6,8). The best advantages of PCR-based methods are the high ability for detection of low amount of *Leishmania* DNA in a variety of clinical samples, such as skin biopsy, blood, bone marrow

Grant sponsor: Tehran University of Medical Sciences, Iran.

*Correspondence to: Mehdi Mohebbali, School of Public Health, Tehran University of Medical Sciences, P.O. Box 14155-6446, Tehran, Iran. E-mail: mohebbali@yahoo.com, mohebbali@tums.ac.ir

Received 13 October 2009; Accepted 17 February 2010

DOI 10.1002/jcla.20377

Published online in Wiley Online Library (wileyonlinelibrary.com).

puncture, or samples prepared from Giemsa-stained slides even in paraffin block. Correct diagnosis and identification of the *Leishmania* species is important for evaluating prognosis and prescribing appropriate treatment (4,9). Although a number of studies have examined the sensitivity and specificity of PCR assays as diagnostic techniques for CL and identification of *Leishmania* species, only a few studies have evaluated these methods on serosity materials that are punctured from CL lesions directly. In this study, we compared the validity of PCR-restriction fragment length polymorphism (RFLP) technique (internal transcribed spacer1 (ITS1)) for DNA detection of amastigote forms of *Leishmania* species in patients with clinical and parasitological confirmed CL.

MATERIALS AND METHODS

Samples Collection

Samples were taken from patients referred to leishmaniasis laboratory in the School of Public Health, Tehran University of Medical Sciences during September 2007 to August 2009. All of the 60 cases were parasitologically confirmed and had typical *Leishmania* lesions acquired during travel or residence in CL endemic areas. Moreover, 10 skin lesions caused by other microorganisms except *Leishmania* parasite, such as *Staphylococcus aureus* ($n = 4$) and *Candida albicans* ($n = 6$), were selected as negative control samples.

Samples Preparation

First, suspected skin lesions were cleaned with 70% ethanol and the samples were collected by injecting 0.2 ml of sterile saline into the dermis of the internal border of skin lesions with 1 ml of sterile insulin syringe with a rotary movement beneath the ulcer bed. After suction, the serosity was transferred to the sterile 1-ml tube containing the 0.5 ml of ethanol 70%. For each patient, two tubes were prepared separately. The tubes were kept in freezer at -20°C until molecular examination. For standard parasitological diagnostic procedure, direct smear was prepared by aspiration of fluid from beneath the ulcer bed. After the smear had dried completely, it was fixed with absolute methanol, allowed to dry again, and stained with Giemsa 10% for microscopic examination.

DNA Extractions

For each patient, DNA was extracted from dermal serosity materials. All DNA extractions were conducted using the FlexiGene DNA kit (Qiagen, Germany) as recommended by the manufacturer.

PCR Amplification

To diagnose and identify the *Leishmania* species isolates, we used the ITS1 region, using the primers LITSR (5'-CTGGATCATTTCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') (Bioneer, Korea) (10,11). Amplification reactions were performed in 30 μl containing 3 μl of PCR buffer (Roche, Mannheim, Germany), 2.5 mM MgCl_2 (25 mM; Roche), 1 μl dNTP (10 mM; Roche), 15 pMol of each primer, 10 ng of genomic DNA and 0.5 units of Taq polymerase (GeNet Bio, Korea). Amplification was performed with 35 cycles, each of 30 s at 94°C , 30-s at 49°C , and 1 min at 72°C in a thermocycler (Biometra, Goettingen, Germany).

RFLP Analysis of Amplified ITS1

The PCR products were digested with *Hae*III (Fermentas, Life Sciences, Germany). Digestion products were separated by using 3% agarose gels in TAE buffer and visualized after staining by ethidium bromide.

Reference Stocks

After electrophoresis, the PCR pattern of samples was evaluated with the pattern of three Iranian *Leishmania* stock species including: *Leishmania tropica* (MHOM/IR/02/Mash10), *Leishmania major* (MRHO/IR/75/ER), and *Leishmania infantum* (MCAN/IR/07/Moheb.gh).

Statistical Analysis

The degree of agreement between PCR-RFLP and parasitological methods was determined by calculating Kappa (κ) values with 95% confidence intervals using SPSS version 13.5 (SPSS Inc., Chicago, IL). Kappa values express the agreement beyond chance and a κ value of 0.21–0.60 represents a fair-to-moderate agreement, a κ value of 0.60–0.80 represents a substantial agreement, and a κ value of >0.80 represents almost perfect agreement (12).

RESULTS

Totally, 60 CL patients, who were parasitologically positive and *Leishman* bodies were demonstrated by microscopically observation ($1,000\times$), were selected. The patient characteristics according to gender, age, number of lesions, ulcers duration, and distribution of the skin lesions on the bodies are shown in Table 1.

Relationship between PCR-RFLP patterns of *Leishmania* DNA with grading of amastigote counts on Giemsa-stained smears is shown in Table 2.

Using the LITSR/L5.8S primer set for PCR, a single product of the expected size (300–320 bp) was amplified from all samples of the *Leishmania* subgenus that was

TABLE 1. The Main Characteristics of 60 Cutaneous Leishmaniasis Positive Patients

Characteristics	
Age (year) ^a	30.3 ± 15.7
<i>Gender</i>	
Male	34(%56.7)
Female	26(%43.3)
Average number ^a of lesions	2.7 ± 2.27
Lesion duration ^a (day)	18.3 ± 76.28
<i>Lesion site on</i>	
Face	15(25%)
Hand	18(30%)
Leg	7(11.7%)
Mix	17(28.3%)
Other sites	3(5%)
<i>Positive results</i>	
Microscopy	60(100%)
PCR-RFLP	51(85%)
<i>Leishmania species identification</i>	
<i>L. major</i>	32(53.3%)
<i>L. tropica</i>	19(31.7%)
Undetermined	9(15%)

^aMean ± SD.**TABLE 2. Relationship Between PCR-RFLP Results with Grading^a of *Leishmania* Amastigote Numbers on Giemsa-Stained Smears**

PCR-RFLP results	Grading of <i>Leishmania</i> parasites				Total
	1+	2+	3+	4+	
Positive	5	12	11	23	51
Negative	6	3	—	—	9
Total	11	15	11	23	60

^aGrading of *Leishmania* parasites was obtained by average parasite density using × 10 eyepiece and × 100 oil-immersion lens as follows: 4+ 1–10 parasites/fields, 3+ 1–10 parasites/10 fields, 2+ 1–10 parasites/100 fields, 1+, 1–10 parasites/1000 fields.

examined. After using the restriction enzyme, banding pattern including the fragments of 220 and 140 bp for *L. major*, the fragments of 200, 80, and 60 bp for *L. infantum*, and 3 fragments of 200, 60, and <60 bp for *L. tropica* were observed in ethidium bromide-stained gels (Fig. 1).

From the 60 microscopy-positive slides, 51 (85%) were positive by ITS1-PCR-RFLP and *Leishmania* species were identified. All the other ten skin lesions were produced by bacterial and fungal agents and they showed negative results. A relative degree of agreement was observed between microscopy and PCR-RFLP assay (61.8%) by kappa analysis ($P < 0.05$).

Figure 1 shows the distribution of *Leishmania* species isolated from the CL patients. The results showed that 19 (31.7%) and 32 (53.3%) of the isolates had

Fig. 1. Restriction fragment length polymorphism (RFLP) patterns obtained from *Leishmania* stocks and patients samples. Lane 1 (*L. major* stock) 2–3, 5–6, 8–9 *L. major*, Lane 4 (*L. tropica* stock) and 7 *L. tropica* M: 50-bp size marker (Fermentas).

patterns similar to *L. tropica*, and *L. major* reference strain, respectively. We could not identify 9 of the 60 *Leishmania* isolates (15%) by RFLP after several times.

DISCUSSION

The parasitological methods are the gold standard for the diagnosis of CL with a high dependence on the number of parasites in samples and requires of technical skills for sampling. The sensitivity of these methods is variable from 27 to 85% for the diagnosis of leishmaniasis and it is the main disadvantage of these methods (13–15). In addition, in culturing methods some problem may affect the results, such as the special requirement materials in culture media for some *Leishmania* isolates and bacterial and or fungal media contaminations. Meanwhile, parasitological methods are not able to identify the species of *Leishmania* isolates. Our main objective for the performance of this survey was to establish a simple and rapid molecular method for early detection of *Leishmania* species on clinical specimens in primary stage of infection and also to treat the infected individuals timely to decrease the morbidity rates of the disease, particularly in ACL form of the disease in the endemic areas of Iran. The ability to identify *Leishmania* species is especially important in prognosis of the disease and in deciding appropriate therapy, especially in regions where more than one type of *Leishmania* species are seen.

In recent decades, different new methods, such as pulsed-field gel electrophoresis and multilocus enzyme electrophoresis, have successively been evaluated for the characterization of *Leishmania* species. Several distinct PCR as well as high-tech and mid-tech molecular assays, such as fluorescence resonance energy transfer (FRET) analysis (16), PCR-RFLP analysis of *Leishmania* HSP70 genes (17), ITS1 (18) and/or single nucleotide

polymorphism analysis of metabolic enzyme genes (19) are available. Different PCR-based methods amplify the repeated nuclear sequences, including ribosomal, mini-exon and repetitive nuclear DNA, or minicircle kDNA. Among the multicopy sequences, sequence of the small subunit of ribosomal RNA gene (SSU rRNA) or 18S rRNA gene is one of the best studied sequences (8,19). Several studies of cutaneous and visceral caused by *Leishmania* parasites have compared the ITS1 and KDNA PCR diagnosis with conventional method. PCR-based method showed more sensitivity than the parasitological methods (8,20). PCR can detect the *Leishmania* DNA parasites in a variety of clinical samples (e.g., skin biopsy, ulcer material, blood, bone marrow and lymph node aspirates). Some studies showed that PCR methods had positive results in more than 90% of CL cases (8,13,20–22).

Despite using PCR assays for detecting *Leishmanial* parasites and being frequently compared with conventional parasitological techniques, rarely have this method been evaluated on serosity materials punctured from CL patients.

In this study, we decided to use ITS1 PCR–RFLP technique for *Leishmania* species identification against serosity materials punctured from cutaneous lesions of patients. Our results showed that there was a relatively good concordance was observed between PCR-RFLP technique and parasitological results, where this molecular technique could detect 51 of the 60 parasitological positive samples ($\kappa = 61.8\%$).

However, this ITS1 PCR-RFLP assay missed 9 of the 60 confirmed microscopy smears and showed 9 false-negative results (sensitivity rate = 85%). Despite DNA extraction from all of the 60 samples, it seems that parasite loads within the skin lesions could have an important role in this investigation. However, the sensitivity of this technique should be improved or it could be used from more sensitive and specific molecular methods, such as nested PCR. We think more satisfactory results would be achieved with some modification in materials and methods and also from using more numerous positive and negative samples.

ACKNOWLEDGMENTS

This research by No: 240/3984 was financially supported by Vice-Chancellors for Education and Research of the School of Public Health, Tehran University of Medical Sciences, Iran. We thank, Mrs B. Akhoundi, Dr. E. Zibafar, Mrs Z. Talaei, Mr H. Falaki. The authors declare that they have no conflict of interest.

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