

## Molecular identification of *Leishmania tropica* infections in patients with cutaneous leishmaniasis from an endemic central of Iran

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**Abstract.** The most common form of the disease is cutaneous leishmaniasis (CL) which is a public health and social problem in many countries especially Iran. In endemic areas where other diseases with similar clinical symptoms occur, definitive diagnosis of CL is very important. The detection and identification of *Leishmania* in infected patients is crucial for achieving a correct treatment and prognosis. To our knowledge, this is the first comprehensive study in terms of geographical distribution and molecular identification of *Leishmania tropica* isolates in central of Iran. This study was performed between 2010 and 2011, during which 218 CL suspected patients referred to Shahid Sadoughi University of Medical Sciences in Yazd, Iran for confirmation were examined. After microscopic analysis, DNA extraction was performed for identification. The molecular target region was *ITS1* gene. Results showed that out of 218 isolates, 102 (46.8%) samples were positive for Leishman body using molecular assay. After PCR-RFLP, analysis identified 50 (49.01%) samples as *L. major* and 52 (50.98%) as *L. tropica*. Two samples showed a different pattern that were reported as unknown. Among *L. tropica*, six different isolates were identified in this endemic area. Finally, this study showed heterozygosity among *L. tropica* isolates in this endemic area such as some other studies from the world. This heterozygosity among the strains may suggest a sexual recombination or genetic exchange between strains.

### INTRODUCTION

The leishmaniasis as a group of neglected tropical diseases caused by genus *Leishmania* remains a public health challenge in more than 80 countries in tropical and sub-tropical areas with considerable as emerging and uncontrolled, and highlighting the need for new and better tools for their diagnosis, treatment and prevention (Desjeux, 2004).

The most common form of the disease is cutaneous leishmaniasis, which is a public

health and social problem in many countries including Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru. Cutaneous leishmaniasis (CL) in the Old World is caused by *Leishmania major*, *Leishmania tropica*, and *Leishmania aetiopica* and *L. infantum* (Nadim & Faghih, 1968).

In endemic area where other diseases with similar clinical symptoms occur, definitive diagnosis of CL is very important. The detection and identification of *Leishmania* in infected patients is crucial

for achieving a correct treatment and prognosis (Schallig & Oskam, 2002; Arévalo *et al.*, 2007).

The gold standard for diagnosis of Leishman body is the parasites isolation and microscopic visualization from a lesion but because of morphological similarities among the species and also different isolates of this genus, common parasitological assessments cannot discriminate between them (Khademvatan *et al.*, 2011; Kobets *et al.*, 2012). Species and strain identification of the etiological agent is only possible with other techniques (Reithinger *et al.*, 2007). Multilocus enzyme electrophoresis (MLEE) (Rioux *et al.*, 1990), which is the gold standard for the identification of *Leishmania* species, requires prior isolation and mass culturing of the parasites. However, isolation can be complicated by the occurrence of secondary infections and the protocol is costly and time consuming.

Techniques based on the DNA analysis have been developed as alternative approaches for the diagnosis of CL and for typing of the *Leishmania* genus (Hajjaran *et al.*, 2011; Sharifi *et al.*, 2012; Khosravi *et al.*, 2012; Eslami *et al.*, 2012; Dabirzadeh *et al.*, 2012). PCR assays targeted at amplification of the internal transcribed spacer 1 (*ITS1*) of rDNA are among the most commonly used methods for the diagnosis and identification of *Leishmania* species in the Old World (Schönian *et al.*, 2010a). Digestion of the *ITS1* amplicon using the restriction enzyme *HaeIII* has been demonstrated to be able to distinguish between nearly all *Leishmania* species (Schönian *et al.*, 2003).

In Iran, one of the important endemic foci for anthroponotic cutaneous leishmaniasis caused by *L. tropica* is Yazd (Yaghoubi-Ershadi *et al.*, 2002). The main objective of this study was to identify the causative *Leishmania tropica* isolates involved in the central endemic area of Iran, Yazd Province. Knowledge of CL identification could help in selection of optimal therapy and treatment regimens. Moreover, this method can be carried out in surveys for detection of cases, reservoir hosts and vectors for better understanding of the transmission mechanisms and control of this complex

disease (Jirku *et al.*, 2006). To our knowledge, this is the first comprehensive study in terms of geographical distribution and molecular identification of *Leishmania tropica* isolates in Yazd Province, Central of Iran.

## MATERIALS AND METHODS

### Patients and samples

This study was performed between 2010 and 2011, during which 218 CL suspected patients referred to Shahid Sadoughi University of Medical Sciences in Yazd were examined. A clinical/epidemiologic data questionnaire was completed for each recruited patient. Ethical clearance for the study was granted by the Ethical Research Committee of the Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

### Isolation and parasite examination

Two microscopic smears were taken from each patient by scraping of the raised internal border of skin lesion(s) by an experienced medical staff. One smear was methanol-fixed and stained with Giemsa for microscopic examination and the other one was used for molecular assay.

### Standard species

Three known strains were used as control in the molecular study; the Iranian reference strain of *L. major* (MRHO/IR/75/ER), *L. tropica* (MHOM/IR/99/YAZ1), and *L. infantum* (MCAN/IR/97/LON49).

### DNA extraction

The slide was soaked in sterile phosphate-buffered saline (PBS; pH=7.4) and the smear on the slide was completely removed with surgical blades and transferred into a sterile 1.5 mL microtube (El Tai *et al.*, 2001), washed 3 times with sterile PBS, and centrifuged at 3,000 rpm for 5 minutes at room temperature. Then, the pellet was resuspended in 200  $\mu$ L of TE buffer (10 mM Tris, 1mM EDTA, pH=8.0), 200  $\mu$ L of binding buffer, and 20  $\mu$ L of proteinase K, and were incubated either at 72°C for 2 hours or at 56°C overnight. Finally, DNA extraction was done using the DNA isolation kit for Cells and Tissues

(Roche, Germany) as recommended by the manufacturer. Extracted DNA was assessed by agarose gel electrophoresis and spectrophotometer, and then was stored at -20°C for further use.

### **PCR amplification of internal-transcribed-spacer 1 (*ITS1*)**

The small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal *ITS1* were amplified using the primers LITSr (5'-CTGGATCA TTTTCCGATG-3') and L5.8s (5'TGATACCAC TTATCGCACTT-3') (Davila & Momen, 2000). Amplification of the DNA was performed in a 50-µL reaction tube containing 0.2 mM deoxyribonucleotide triphosphates (dNTPs) mix, 1.5 mM Magnesium Chloride (MgCl<sub>2</sub>), 1 U of Taq DNA polymerase (Fermentas, Leon-Rot, Germany), 10 pmol of each primer, and 100-1000 ng of extracted DNA obtained from the isolates. Amplification stages were as follows in a Veriti™ thermal cycler (ABI, USA): initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 45 seconds, the annealing at 50°C for 45 seconds, and the extension at 72°C for 45 seconds, with an additional and final extension at 72°C for 5 minutes. Five micro liters of PCR product were run along with a 50-base pair (bp) DNA ladder on a 1% agarose gel containing DNA Green viewer at 5 V/Cm. The PCR products were analyzed under a UV transilluminator, and were evaluated in comparison with the 3 *Leishmania* standards.

### **Species analysis**

The PCR products were digested using restriction enzyme *Hae*III (Fermentas, Leon-Rot, Germany) at 37°C for 1 hour to analyze the species. The digested fragments were assessed using 2% agarose gel in 0.5X TBE buffer and visualized through staining with DNA Green viewer under a UV transilluminator. The *L. tropica* isolates were collected for further molecular analysis.

### **Identification of *L. major* isolates:**

The PCR products regarding to *L. major* isolates, were digested using restriction enzymes of *Taq*1, *DPN*1 and *Alu*1

(Fermentas, Leon-Rot, Germany) at 37°C for 1 hour. The digested fragments were assessed using 2% agarose gel in 0.5X TBE buffer and visualized through staining with DNA Green viewer under a UV transilluminator. The *L. tropica* isolates were collected for further molecular analysis.

### **Statistical analysis**

The number of positive clinical samples was calculated as a percentage using the results obtained for the different molecular markers evaluated in this study for detection of *Leishmania* DNA.

## **RESULTS**

In this study, among 218 isolates obtained from patients with suspected cutaneous leishmaniasis, 102 (46.8%) samples were positive for Leishman body using molecular assay. The most common clinical forms of the lesion for PCR-positive persons were papule and ulcer. All of the PCR-positive cases were Iranian patients, except for 13 patients who were Afghan nationals. Locations of lesions were 47.7% in hands, 30.7% in face, 15.4% in feet, and the remainder in other regions. In 90% of PCR-positive cases, the lesion had occurred during the last month, and about 75% of the patients were living in urban areas. The observed lesions were in different sizes and shapes, including acute necrotizing, ulcerative, nodular, volcano-shaped, impetiginous, erysipeloid, eczematoid, verrucous, tumoral, recidivans, abortive and lupoid. Out of 102 positive samples which were confirmed by *ITS1*-PCR method, 100 (98.03%) cases exhibited the amplicon with 350 bp in length (the presence of which confirms the genus as *Leishmania*), and 2 (1.97%) samples showed apparent variability as an *ITS1*- PCR product was 100 bp different from the standard specimens, since the amplicon was 450 bp in length (Fig. 1).

The *ITS1*-PCR amplicons were digested by *Hae*III. Results identified 50 (49.01%) samples as *L. major* (post restriction enzyme digestion showed 210 bp and 140 bp fragments) and 52 (50.98%) as *L. tropica*

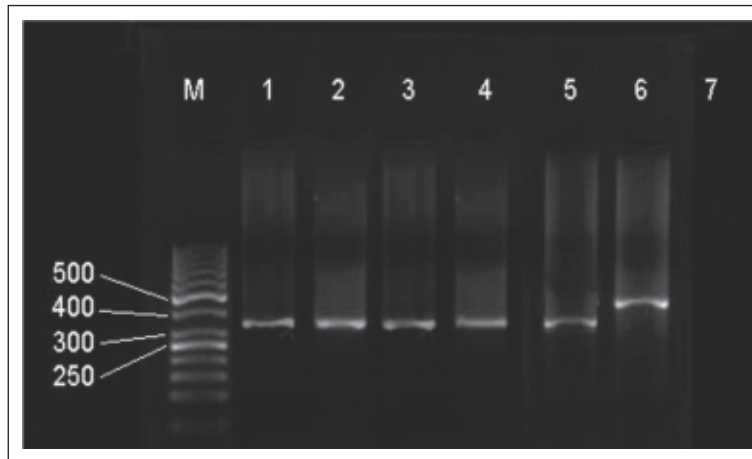


Figure 1. Electrophoresis results of ITS1-PCR from smears. M: Molecular marker (50 bp); Lane 1: Standard *Leishmania major* (MRHO/IR/75/ER); Lane 2: Standard *Leishmania tropica* (MHOM/IR/99/YAZ1); Lane 3: *Leishmania infantum* (MCAN/IR/97/LON49); Lane 4, 5: PCR product according to standard sample; Lane 6: PCR product with no accordance with standard sample; Lane 7: negative control.

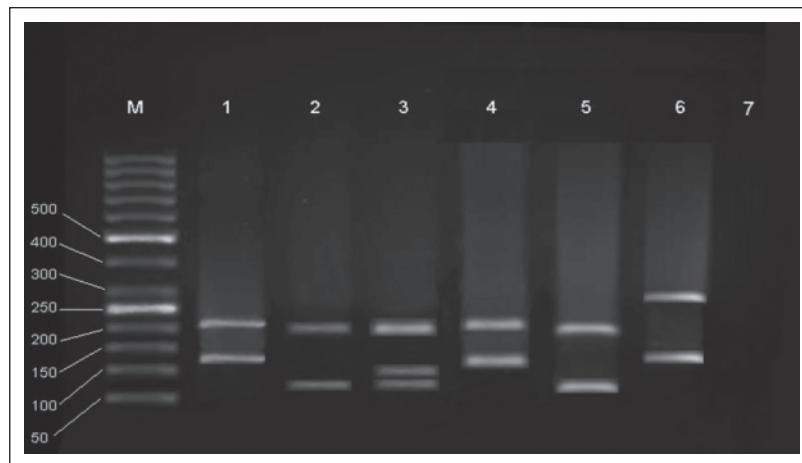


Figure 2. Restriction enzyme digestion profile of amplified *ITS1* region with the restriction enzyme of HaeIII. M: Molecular marker (50 bp); Lane 1: Standard *Leishmania major*; Lane 2: Standard *Leishmania tropica*; Lane 3: Standard *Leishmania infantum*; Lane 4: *Leishmania major* according to standard; Lane 5: *Leishmania tropica* according to standard; Lane 6: The one sample different from the normal samples and standard patterns; Lane 7: negative control.

(post restriction enzyme digestion showed 200 bp and 60 bp fragments) (Fig. 2). After restriction enzyme digestion, two (1.97%) samples showed a different pattern compared to all the standards, since it had fragments of 153 bp and 296 bp (Fig. 2). This

sample was sent for sequencing, and the result was assessed using BLAST software. Among the 102 positive samples, six different isolates were identified after digestion of *ITS1*-PCR amplicon with *TaqI* restriction enzyme (Table 1).

Table 1. Digestion of *ITS1* amplicon with *TaqI*. Six different patterns were made after enzyme digestion

Group	Fragment's size	Number of cases
A	200, 190, 128, 110	19
B	190, 128, 110	22
C	220, 200, 110	4
D	180, 128	1
E	180, 105	1
F	210, 190, 130	2

## DISCUSSION

We identified the causative agent of anthroponotic cutaneous leishmaniasis, *Leishmania tropica* at one of the important epidemic area inside the central region of Iran based on the *ITS1*-PCR-RFLP, an approach proven to be useful for the molecular identification of *Leishmania* species. The differential diagnosis of species and strains as well as suitable therapeutic strategies for the disease can be determined through precise protocols for identification of isolates from endemic areas where more than one *Leishmania* species is present. This information can be used for ecological, clinical, and epidemiological studies on *Leishmania* species. Our findings confirmed that around half of the clinical specimens with cutaneous leishmaniasis have the agent of *L. tropica*, which shows six different patterns based on RFLP analysis. Identification of *L. tropica* has been performed previously in endemic area of Iran (Ghasemian *et al.*, 2011; Khademvatan *et al.*, 2011; Shirian *et al.*, 2012; Khosravi *et al.*, 2012; Sharifi *et al.*, 2012; Kheirandish *et al.*, 2013) but our study is the first report of identification of *L. tropica* from a central endemic area from Iran. This heterozygosity of the *L. tropica* isolates has also been suggested in previously study (Odiwuor *et al.*, 2012). Schwenkenbecher *et al.* (2004) developed sixteen polymorphic micro-satellite markers for phylogenetic analysis

of *Leishmania tropica*. They found a high degree of allelic heterozygosity among the strains and therefore suggested a sexual recombination within the species *L. tropica* (Schwenkenbecher *et al.*, 2004). Mauricio *et al.* (2006) showed this heterozygosity and attributed it to the genetic exchange between strains. Also, this phenomenon has previously been observed in other *Leishmania* species (Schwenkenbecher *et al.*, 2006; Zemanova *et al.*, 2007; Lukes *et al.*, 2007; Rougeron *et al.*, 2009).

We also found that two isolates showed different amplification pattern with a fragment of 450bp comparison with others with 350bp after *ITS1*-PCR. This substantial difference in the PCR product sizes directed us to sequence some of the products. Molecular analysis by BLAST software showed that the selected sequence had a close similarity with *Crithidia fasciculata* (97%), and *Crithidia luciliae* (90%). Based on the recent report by Eslami *et al.* (2012), some isolates obtained from clinical samples with cutaneous leishmaniasis had some genes that were similar to *L. major* and *Crithidia* species, suggesting possible genetic hybridization between two different genuses, *Leishmania* and *Crithidia*, that are so distant phylogenetically and epidemiologically. In the New World, more evidences for hybridization events have been reported (Darce *et al.*, 1991; Banuls *et al.*, 1997). The hypothesis that certain *Leishmania* genotypes correspond to hybrid genotypes between different species in the Old World has been first proposed by Evans *et al.* (1987). Some other studies have also reported this similarity before (Doudi *et al.*, 2010; Eslami *et al.*, 2011).

In conclusion, the *ITS1* PCR-RFLP enabled us to detect and to identify *L. tropica* in clinical samples obtained from patients with cutaneous leishmaniasis. We have demonstrated that *L. tropica* in this endemic area has six heterogeneity patterns. This study should be followed by some other studies at different loci from around this endemic region.



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