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Original Article

Molecular Identification of *Leishmania* Species Isolated from Human Cutaneous Leishmaniasis by RAPD-PCR

*S Alimoradi [1, 2], *H Hajjaran [1], M Mohebali [1], F Mansouri [2]*

[1]*Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran*
[2]*Health Center of Kermanshah Province, Iran*

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Abstract

Background: Characterization of *Leishmania* parasites is necessary for epidemiological objectives such as documenting the distribution of predominant species and designing appropriate control measures. In this study, we aimed to identify *Leishmania* species isolated from cutaneous leishmaniasis (CL) patients, using RAPD-PCR method.

Methods: This descriptive, cross-sectional study was designed against 20 *Leishmania* spp. which were confirmed by parasitological examination, isolated from 30 suspected cutaneous leishmaniasis patients, referred to Health Centers of Kermanshah Province from August 2006 to December 2007. All desirable samples were characterized by RAPD-PCR method using five selected oligoprimers (AB1-O7, A4, 327, 329 and M13). Electrophoresis patterns from each isolate were compared with reference strains of *L. tropica, L. major* and *L. infantum.*

Results: Eighty nine percent and 11% of isolates were similar to *L. major* and *L. tropica* reference strain, respectively. Five of the isolates were identified as *L. major* using RAPD-PCR, induce ulcers at the base tail of Balb/c mice, 4-6 months after inoculation.

Conclusion: *L. major* is dominant in the studied areas and it seems that some parts of the Kermanshah Province to be probably considered as zoonotic cutaneous leishmaniasis areas in the middle west of Iran.

Keywords: *Cutaneous Leishmaniasis,* Leishmania *species, RAPD- PCR, Iran*

Introduction

Leishmaniasis is one of the most important vector-borne disease, and it is estimated that there are 1.5 to 2 million new cases on annual, with up to 350 million people at risk of infection and disease (1).

Parasites belonging to the genus *Leishmania* cause different cutaneous, muco-cutaneous and visceral infection (2).

Leishmaniasis, is a major public health problem in at least 15 of 30 provinces of Iran. There are at least 2 species of old world *Leishmania*, are responsible for self healing ulcerative disease and atypical infections (3). *L. major* is causative agent of zoonotic cutaneous leishmaniasis (ZCL) and *L. tropica* causes anthroponotic cutaneous leishmaniasis (ACL). ZCL is endemic in central, north east, southeast, south west and west of Iran and ACL has also seen as an endemic disease in some of the largest cities of Iran (4).

CL cases have been increased in some parts of Kermanshah province in recent years and majority CL cases (25%) were reported from Ghasre-Shirin and Sarpole-Zahab which are closed to Iraq border (5). As distribution of *Leishmania* species in Kermanshah Province particularly in the cities was unknown and few endemic foci of CL were being informed in some parts of this province thus, a cross-sectional study was designed to identify of *Leishmania,* spp. isolated from cutaneous leishmaniasis patients referred to some of Health Centers of Kermanshah Province, using RAPD-PCR method

Essentially, *Leishmania* species have similar morphology and sometimes cause similar clinical manifestation; therefore, differentiations among species require molecular or biochemical techniques such as PCR or Isoenzymes analysis (6). Biochemical methods are lengthy, complicated and expen-

* **Corresponding Author:** Tel: +98 21 88951392; Fax: +98 2166462267, E-mail: hajaranh@tums.ac.ir

sive procedure. It is shown Random Amplified Polymorphic DNA (RAPD) technique is capable of discriminating between species of *Leishmania*. RAPD uses random oligomers to amplify genomic DNA and thus does not necessitate any prior knowledge on organism's genomic sequences (7).
Distribution of *Leishmania* species in Kermanshah Province particularly in the cities was unknown and a few endemic foci of CL were being informed in some parts of this province thus, a cross-sectional study was designed to identify of *Leishmania* spp. isolated from cutaneous leishmaniasis patients referred to some of Health Centers of Kermanshah province, using RAPD-PCR method.

Material and Methods

Study area
This study was carried out in some cities of Kermanshah Province where majority cutaneous leishmaniasis cases had been reported in recent years (5). Kermanshah has a moderate and mountainous climate. The population of this province is 1,938,060. The studied CL cases presented with a clinical diagnosis of cutaneous leishmaniasis and also *Leishman* bodies or amastigotes were seen in microscopically dermal lesion smears. Patients with secondary infection particularly bacteriological and fungal infections were excluded from this study.

Sample collection and culture
Samples were taken from skin lesions of 30 suspected CL patients with 1.5-60 yr age that referred to the Health centers or private medical diagnosis laboratories in different areas of the province. Samples were collected from CL patients including; Ghasre-Shirin-8 cases, Sarpole-Zahab-13 cases, Harsin-2 cases, Islam-Abadegharb-6 cases and Kermanshah-1 case (Table 1). Samples were collected by a sterile vaccino style from the swollen edge of the skin lesions of cases and thin smears were prepared. Smears were fixed in methanol, stained by Giemsa. Also, samples were cultured in N.N.N medium and sub-cultured into Schneider's medium with 10% Fetal Bovine Serum (FBS) and 200u/ml penicillin G incubated at 26° C (7).

Animal inoculation
Some *Leishmania* isolates were harvested at stationary phase and 2×10^6 promastigotes were inoculated subcutaneously into the base of the tail of Balb/c mice. Animals were examined weekly for appearance of lesion at the injection site up to 6 months.

DNA extraction
DNA was extracted from cultured promastigotes by phenol- chloroform extraction method according to Kelly method (8). The DNA samples dissolved in 50 µl DDW and stored at 4º C.

RAPD-PCR Procedure
Amplification reactions were preformed according to described earlier by Mauricio et al (7, 9). The following 5 arbitrary primers were used to generate random DNA markers, (AB1-07): ggt gac gca g, (A4): aat cgg gct c, (327): ata cgg cgt c, (329): gcg acc ctc c, (M13): gta aaa cga cgg cca gt. Each 20 µl of RAPD reaction contained 20 ng genomic DNA, 3.0 mM Mgcl2, 0.2 mM dNTP, 20 pmol of each primer, 1u of Tag DNA Polymerase (Roche Biotech) in the PCR buffer .The cycling conditions were 94º C for 5 min, followed by 35 cycles of 94º C, 37º C, 72º C for 1 min each, and 1 cycle of 72º C for 10 min in Thermocycler (Techne USA). PCR products were loaded onto a 1.2% agarose gel containing ethidium bromide for 4 h at 50V. After gel electrophoresis, the patterns of each isolate was evaluated with three *Leishmania* standard species including *L. major* (MRHO/IR/75/ER), *L. tropica* (MHOM/IR/02/ Mash-2) and *L. infantum* (MCAN/ IR/97/LON49).

PCR amplification
In different experiments, LITSR (5'ctggatcattttccgatg3') and L5.8S(5' tgataccacttatcgcactt 3') primers was used to amplify ITS1 region of ssu rDNA from 1 *L. tropica* and 1 *L. major* isolates from Kermanshah and Ghasr- Shirin Cities respectively. ITS1 amplification reactions were preformed following the protocol described earlier. Briefly, 25

µl PCR reaction containing 10ng of total genomic DNA, 10 pmol of each primer, 0.2mM each dNTP, 2.0 mM Mgcl2 and 1U of Tag polymerase in the PCR buffer. The cycling conditions were 95° C for 5 min, followed by 35 cycles of 94° C for 30s, 50° C for 30s, and 72° C for 1 min in a Techne USA thermocycler (10, 11).

DNA sequencing

DNA for sequencing was prepared by the ITS1-PCR. The products of 2 *L. tropica* and *L. major* isolates were sequenced at Kawsar Biotechnology Center, Tehran, Iran and MWG (mwg_ biotech.com,) Germany. Nucleotide sequence data have been submitted to the GenBank database with Accession No, EU482829 for *L. tropica* and EU482830 for *L. major* (12).

Results

Thirty cases of suspected cutaneous leishmaniasis were examined by parasitological exam, 20(66.6%) cases of them were parasitological positive, Leishman bodies were observed by microscopically observation (1000X) and promastigotes developed in culture media. From 20 confirmed parasitological cases, 13(65%) were male and 7(35%) of them were female. The most highly infected age group mean was 30 yr and the lowest rate was1-10 yr (1 case) and 41-50 yr (1cases). In these parasitological positive cases, 17(85%) had 1-2 lesions, 2(10%) had 3-4 lesions, 1(5%) had 5-6 lesions. Duration of the skin lesions was 3-6 months with the average duration 18 weeks. Of all lesions, 54%, 33% and 13% of them were located on the hands, face and legs respectively. All 20 desirable samples were characterized by RAPD-PCR using five primers: AB1-07, A4, 327, 329 and M13.

The PCR products showed different profiles and amplified DNAs including strong, weak and fuzzy bands. The number of bands amplified in each strain varied between five to 10 and the size of them ranged between 100bp to 1500bp. The analysis of the results of discriminative bands in electrophoresis pattern for *L. tropica*, *L. major* and *L. infantum* Stocks and the examined isolates obtained with the primers are illustrated in Table 2 and Fig.1 (A1: primer AB1-O7, A2: primer A4, B1: primer 327, B2: primer 327, C: primer M13 primer).

So by comparing the pattern of electrophoretic profile of the studied isolates with the electrophoretic pattern of reference strains identified that from 20 isolates, the prevalence of *L. major* was 17 (85%) and 3 (15%) was *L. tropica* among the cases. *L. major* isolates were from Ghasre shirin, Sarpole-Zahab, Harsin and Islam-Abadegharb cities and *L. tropica* isolates were from Sarpole-Zahab and Kermanshah cities (Table 1).

Five isolates were identified as *L. major* by each 5 Primers and RAPD-PCR, produced lesions, 2 to 4 months after inoculation in the base tail of mice and also, after autopsy the mice, it was seen that the amastigotes disseminate to the other organs and visceralized. Moreover, the sequence of two isolates with accession number EU482829 for *L. tropica* and EU482830 for *L. major* was searched in GenBank for similar sequences with the BLAST program and a strong homology was detected with the ITS1 rDNA Iranian *L. tropica* EF653267 and *L. major* EF653269 isolates in BLAST (12). In this study, *L. tropica* sequenced isolate belongs to a 60 yr woman was infected to Lupoid or recidivan form of cutaneous leishmaniasis for more than two years (Fig. 2).

Table 1: Characterization of *Leishmania* spp isolated from human skin lesions in some cities of Kermanshah province by RAPD-PCR (2006-2007)

City	No. of samples	No-of Characterized isolates	RAPD-PCR Results	
			L. major	*L. tropica*
Ghasre-Shirin	8	7	7	-
Sarpole-Zahab	13	6	4	2
Harsin	2	1	1	-
Islam-Abade Gharb	6	5	5	-
Kermanshah	1	1	-	1
Total	30	20	17	3

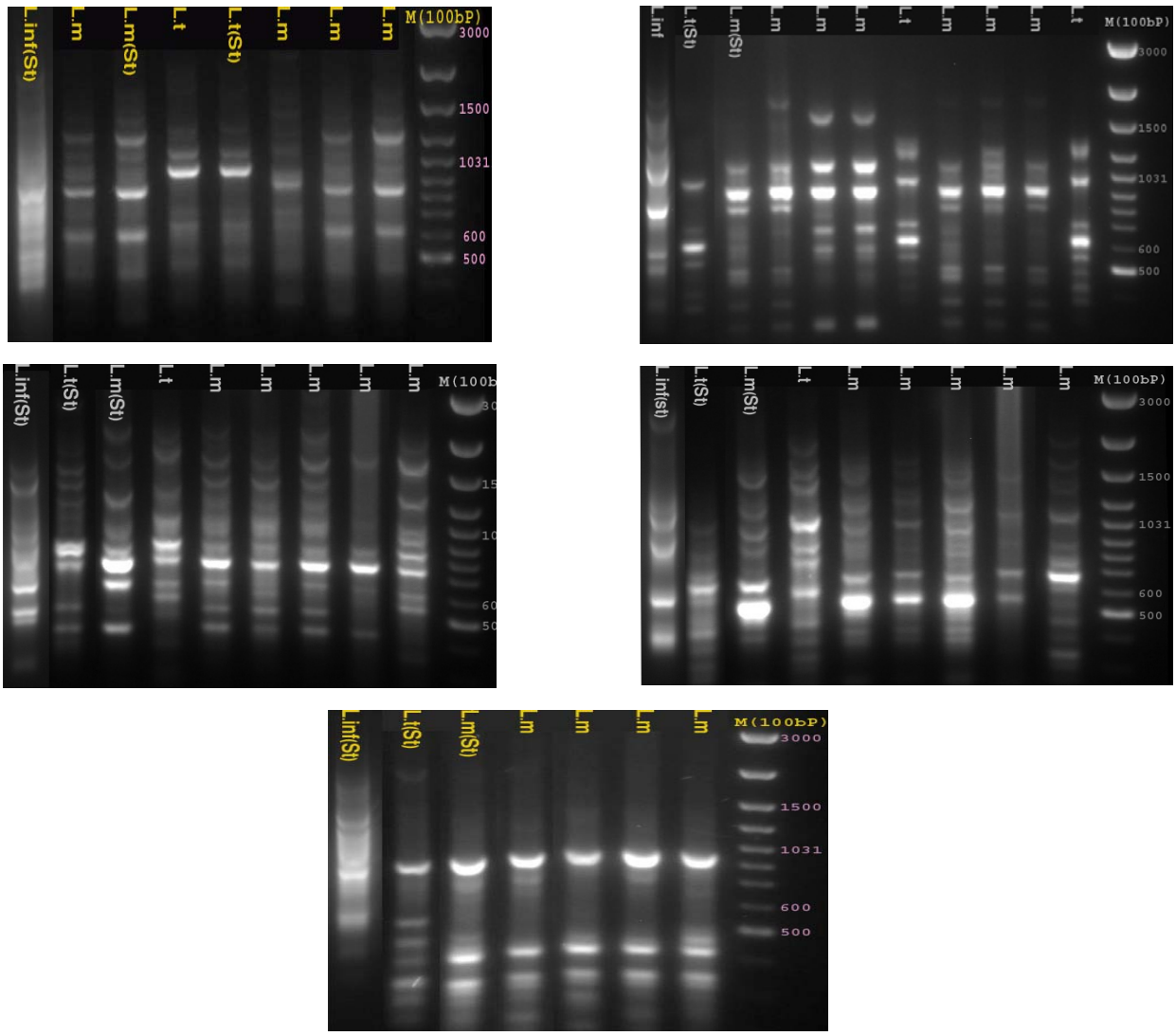

Fig. 1: Random amplified polymorphic DNA (RAPD) amplification profiles observed with AB1-07 (A 1), A4 (A2), 327 (B1), 329 (B2) and M13 (C) primers.
The Figure illustrates different levels of sharpness. Each have corresponds to a DNA isolate, which are identified comparing profiles obtained from *leishmania* reference. M: 100bp size Marker (Ferments).

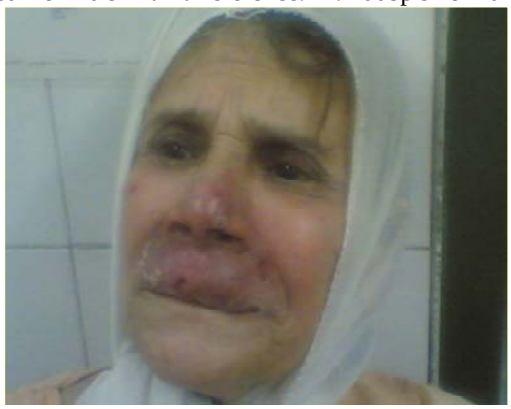

Fig. 2: Cutaneous leishmaniasis patient was infected to Lupoid or Recidivan form with *L. tropica*. Kermanshah city .2007

Table 2: Molecular weight of discriminative bands (in kilo base pair) with different species and primers

Species	Size estimated (in kbp) of RAPD products with primers				
	AB1-O7	**A4**	**327**	**329**	**M13**
L. tropica	0.95, 1.1	0.75, 0.9	0.55, 0.6	0.65, 1.1	0.5, 0.6
L. major	0.6, 0.8, 1.1	0.7.0.8, 0.9	0.8, 0.9, 1.1	0.55, 0.7	0.5, 0.6, 0.9
L. infantum	0.8, 0.85	0.6, 0.7, 0.8	0.6, 0.8, 0.1	0.6, 0.9, 0.1	0.85, 0.9

Discussion

Accurate identification of the *Leishmania* species seems to be necessary for a variety of clinical and epidemiological reasons for deciding distinct treatment regimens and also designing appreciate control programmers (13). DNA- based techniques have commonly been used as potential tools for this purpose (14). RAPD does not require previous knowledge of nucleotide sequences. In RAPD analysis, only one randomly designed decamer with 60-70% GC were used (15). Based on previous experiments (7, 16, 17) we used RAPD-PCR methods for characterization of cutaneous *Leishmania* isolates from Kermanshah Province. Until now, the etiology of CL and predominant of *Leishmania* species has been unknown in Kermanshah Province.

In the current study, the prevalence of CL was in aged group of 1.5 to 60 yr with the average age 30 yr, and in the 68% cases the site of skin lesions were on the hands and legs or lower part of the body. From 20 characterized isolates, the prevalence of *L. major* was 85%, these isolates were from Ghasre-Shirin, Sarpole-Zahab, Islam-Abade Gharb and Harsin Cities and *L. tropica* was 15% and these isolates were from Sarpole- Zahab and Kermanshah Cities. Recidivan or Lupoid form isolate was from Kermanshah City. Five isolates were already determined as *L. major* could produce ulcer at the base tail of Balb/c mice. Therefore, a strong correlation is observed between the results of RAPD-PCR and animal inoculation.

In conclusion, characterization of *Leishmania* isolates collected showed that *L. major* is predominant agents of CL, and like other western region. On the basis of studies were done in the south west and west of Country in Khuzestan and Ilam Provinces, *L. major* isolates were recovered and identified from CL patients (18, 19) and *L. major* recovered from wild rodents like *Tatera indica, Meriones libycus* as the principal reservoirs host in this area (20).

In the basis of preliminary, this study revealed that *L. major* are dominant in the studied areas and it seems some parts of the province to be probably considered as ZCL areas in the middle west of Iran. Further studies based on reservoir, sand-fly as the vector with a greater number of cutaneous leishmaniasis cases must be carried out in order to clarify the epidemiological aspect of leishmaniasis in the province. RAPD method in spite of some draw backs is a suitable and powerful tool for characterization of *Leishmania* species (21, 22).

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