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Identification of *Leishmania* Species Isolated from Human Cutaneous Leishmaniasis, using Random Amplified Polymorphic DNA (RAPD-PCR)

H Hajjaran [1],*M Mohebali [1], MR Razavi [2], S Rezaei [2], B Kazemi [3], GhH Edrissian [1]
J Mojtabavi [4], B Hooshmand [5]

[1]Dept, of Parasitology and Mycology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran
[2]Dept, of Parasitology, Pasteur Institute of Iran, Tehran, Iran
[3]Dept, of Medical Parasitology, School of Medicine, Shaheed Beheshti University of Medical Sciences, Iran
[4]Health Center of Khorasan Province, Mashhad, Iran
[5]Dept. of Disease Control. Ministry of Health and Medical education Tehran, Iran

Abstract

This cross-sectional study was designed to isolate of *Leishmania* spp from cutaneous leishmaniasis patients and characterized them by RAPD-PCR technique. Eighty- seven *Leishmania* isolates from 112 samples were collected from cutaneous leishmaniasis (CL) patients who referred to Mashhad Health Centers from August 2002 to May 2004. Desirable samples (87 isolates) were characterized by RAPD-PCR method using four selected oligoprimers. Electrophoresis patterns from each isolate were compared with reference strains of *L. major, L. tropica and L. infantum.* The results showed that 94.2% and 5.8% of isolates were similar to *L.tropica and L.major* reference strain, respectively. Four isolates that were determined by RAPD-PCR as *L.major,* could produce ulcer at the base tail of BALB/c mice, 4 - 12 weeks after inoculation but none of *L. tropica* isolates produced any lesions at the site of injection in the animals. The results indicate that *L. tropica* species are dominant in the studied areas of Mashhad city and RAPD-PCR technique is a suitable tool for *Leishmania* characterization in epidemiological studies.

Keywords: Leishmania major, Leishmania tropica, *Cutaneous leishmaniasis, RAPD-PCR, Iran*

Introduction

Leishmaniasis is an important public health problem in tropical and subtropical countries (1). Human cutaneous and visceral leishmanias i*s* both occur in some parts of Iran (2). Leishmaniasi*s* is associated with a variety of clinical manifestations, depending on the species of the parasite, the host immune response and factors in the saliva of the sand fly vector (1). In Iran, there are at least 2 species of old world *Leishmania*, which are responsible for self-healing ulcerative disease and occasionally metastatic cutaneous leishmaniasis (2). In addition, there are reports of atypical infections, which constitute exceptions to the rules (3). In our country, *L.major* is causative agent of zoonotic cutaneous leishmaniasis (ZCL) and *L.tropica* causes anthroponotic cutaneous Leishmaniasis (ACL). ZCL is endemic in central, northeast, southwest and southeast parts of Iran (2), whereas ACL has been an endemic disease in some large and medium sized cities of our country such as Mashhad, Tehran, Shiraz, Kerman, Bam and Yazd (4). Cutaneous leishmaniasis is considered as an old endemic disease in many parts of Khorasan province, in the northeastern Iran (5). The reported CL cases have been increased in some parts of Mashhad

***Corresponding author:** P.O. Box: 14155-6446, Fax: 6462267, E-mail: mmohebali@hotmail.com

city from 2000 to 2002, although, a total of 4900 CL cases were officially reported in the city but this frequency of the disease is probably a big underestimate. In recent years, ACL has become the most important endemic disease in this city and a matter of concern for health authorities.

Essentially, characterization of *Leishmania* parasites is necessary for epidemiological objectives such as documenting the distribution of prevalent species and designing appropriate control measures (6). Microscopic examination of clinical materials or cultured parasites is not adequate for species identification due to morphological similarity of different species and alternative methods such as isoenzyme analysis, immunological approaches with monoclonal antibodies and classical DNA-based techniques, e.g. restriction analysis of kinetoplast DNA (KDNA) technique are lengthy, complicated and expensive procedures and require large-scale cultivation of parasites and sophisticated laboratory setting. Certain monoclonal antibodies appear to be promising for species identification by serodeme analysis and the reactivity patterns of some species vary significantly depending on the geographical origin of the parasites and they do not access for all *Leishmania* species (7-11). Recently it has been shown that Random Amplified Polymorphic DNA (RAPD) is capable of discriminating between species of *Leishmania* (12-14). RAPD technique uses random oligomers to amplify genomic DNA and thus does not necessitate any prior knowledge on organisms' genomic sequences. It has been used to study polymorphism of microorganisms of medical important such as *Trypanosoma* (15) and *Plasmodium* species (16). Thus, in this study we used RAPD-PCR as a rapid, sensitive, specific and low-cost alternative technique for *Leishmania* species determination.

Materials and Methods

Study area This study was carried out in Mashhad city from August 2002 to May 2004, where cutaneous leishmaniasis has long been known as an endemic disease (2). Mashhad, city as the center of Khorasan province is located in the northeastern Iran with a population of more than 2.7 millions.The populations have been increased to 110 percent from 1976 to 2002 and the growth is attributed to the large number of Afghan refugees who constitute a population of approximately 450000(17). The sample was collected from patients who were referred to Mashhad Health Centers in various parts of the city.

Sample collection and culture Patients with skin lesions were selected for the study. After obtaining a complete clinical history, the samples were taken from the swollen edge of the lesion by a vaccinostyle. A direct smear for microscopical examination was prepared and the samples were also cultured into liquid phase of Novy-MacNeal and Nicole(NNN) medium (18) and Schneider's medium (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and 200u/ml penicillin G. For mass production, primary *Leishmania* isolates were subcultured in RPMI 1640 (Gibco) media with 10% FBS at 26^{o}C.

Animal inoculation All *Leishmania* isolates were harvested at stationary phase of growth from above media and then about $2x10^{6}$ promastigotes were inoculated subcutaneous into the base of the tail of 47 groups of Balb/C mice. Each group consisted of three mice, which were examined weekly for appearance of lesion at the injection site up to 6 months.

Preparation of total genomic DNA Promastigotes from a 15 ml stationary phase of bulk culture were harvested by centrifugation (3000g at 4 ^{o}C for 10 min) and washed 3 times in cold sterile PBS (pH 7.2). The pellet was resuspended in 500 µl of cell lysis buffer (50mM Nacl, 50mM EDTA, 1%SDS, and 50mM Tris-HCl, pH 8.0) with 100 microgram /ml ProteinaseK, and incubated at 55^{o}C overnight. The lysate was extracted by phenol /chloroform followed by ethanol precipitation (19). The DNA was resuspended in double distilled water

(DDW) and stored at 4°C. Working solutions were adjusted to 10ng/ul in DDW (20).

RAPD-PCR procedure Amplification reactions were performed following the protocol described earlier (21). Each 20 µl of RAPD reaction contained 20 ng genomic DNA, 2.0 mM $MgCl_2$, 0.2 mM dNTP (Roche Biotech), 20 pmol of each primer, 1u of *Taq* polymerase (Roche Biotech) in the PCR buffer. Reactions were overlaid with 30 ul of mineral oil and amplified in a thermocycler (Techne USA) programmed for one cycle at 94°C for 5 min followed by 35 cycles of 94ºC, 36ºC, 72ºC for 1 min each, and 1 cycle of 72ºC for 10 min. Twelve µl of PCR products were run along with a 100 bp ladder on a 1.2% agarose gel containing ethidium bromide for 4 h at 50V. The gel was observed on a UV transilluminator and then, digital photographs were prepared. Also, we tested 12 decamer primers and selected the suitable ones (14). Twelve primers (Roche Biotech) were evaluated with three *Leishmania* standard species including *L.major* (MHOM/IR/75/ER), *L.infantum* (MCAN /IR/97 /LON 49) and *L.tropica* (MHOM/IR/99). Four out of 12 primers that produced bright and consistent bands were selected for this investigation (Table 1).

Results

RAPD-PCR method was carried out by 4 selected primers (AB1-07, A4, 327, 329) (Table-1) for identification and characterization of 87 human CL isolates collected from different parts of Mashhad city (Table2).

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 one to twelve and the size of them ranged between 0.3kb to 2.4 kb. Two criteria were taken into account: the consistent presence of amplified bands at the same electrophoretic position for isolates of DNA samples' concentration was quantified by both running them on an agarose gel along with a known concentration of DNA and UV spectroscopy. a same species and the discrimination between isolates belonging to different species. This allowed analysis of the results obtained with the primers AB1-07, A4, 327 and 329, these profiles are illustrated in Fig.1 (with AB1-07 primer), Fig.2 (with A4 primer), Fig. 3 (with primer 329), and Fig. 4 (With primer 327)Table 3 summarizes the electrophoretic position of the diagnostic bands identified for each primer and species tested.

Two major DNA products were observed in *L.tropica* stock (No.9), which appeared for most species with the primer AB1-07 (Fig.1). The sizes were consistently estimated to be 0.9 and 1.05 kb (marker 100bp), so by comparing unidentified *Leishmania* isolates, with those obtained from ref.strains, samples Nos.1, 2, 4-6, 9, 11-13 were diagnosed as *L. tropica; L.major* could produce fragments of 0.4 to 2kb (No.10). So by comparing unidentified *Leishmania* isolates, with those obtained from ref. strains, samples Nos. 3, 7, 8 were diagnosed as *L.major*.

With A4 primer (Fig.2) 2 bands of 0.7 and 0.85 kb size and bands with size of 0.3 to 2.4 kb and 0.8, 1.05 kb were consistently observed for *L. tropica* (No.2), *L.major* (No.8) and *L.infantum* (No.1) stock, respectively. So by comparing unidentified *Leishmania* isolated with those obtained from stock, isolates Nos. 2 to 7 and 9 were *L.tropica*.

In the case of primer 329 (Fig .3), discriminative bands of 5kb and 2.2 kb consistently were observed for *L.tropica* (No.5) stock, fragments 0.2 to 2kb for *L.major* (No.4) and 0.65, 0.7kb as well as 0.75 kb for *L.infantum* (N0.1). So by comparing, samples Nos.2, 3, 6 were *L.tropica*.

In the primer 327 (Fig .4) a band of 1.7 kb, bands of 0.5 to 2.4 kb and two bands of 0.95 & 1.0 kb allowed discrimination among *L.tropica* (No.8-A), *L.*major (No7-A) and *L.infantum* (No.4-B). So by comparing, in Fig.4 a samples 1 to 6 and 9 to 13 were *L.tropica* and in Fig.4B No.1 to 3 were *L.tropica*.

Susceptibility of Balb/C mice to parasite isolates in groups of three Balb/C mice inoculated

with stationary growth phase of isolate was followed up. After incubation time, groups 5 of mice showed lesions in the site of inoculation. These isolates were determined previously by RAPD-PCR as *L.major.* None of *L.tropica* isolates inoculated with same procedure, produced infection in the mice.

Table 1: The oligoprimers used in this study

No.	Code	Sequence (5- 3)	%G/C
1	A1	CAGGCCCTTC	70
2	A4	AATCGGGCTG	60
3	A8	GTGACGTAGG	60
4	AB1-O7	GGTGACGCAG	70
5	AB1-12	CCTTGACGCA	60
6	AB1-15	GGAGGGTGTT	60
7	AB1-14	TTCCCCCGCT	70
8	AB1-18	CCACAGCAGT	60
9	AB0-01	CCGTCGGTAG	70
10	AB1-09	TGGGGGACTC	70
11	327	ATACGGCGTC	60
12	329	GCGAACCTCC	70

Table 2: Characterization of *Leishmania* parasites isolated from human lesions in some parts of Mashhad by RAPD-PCR.

Location	No. of isolates	RAPD Results	
		L.tropica	*L.major*
Ab/O/Bargh	35	33	2
Hemat Abad	24	22	2
Torghabeh	5	5	-
Vakilabad	5	5	-
Haj-Norouz	9	9	-
Kale-zarkesh	1	-	1
Vali-ye-asr	1	1	-
Khaje-Rabie	4	4	-
Sakhteman	2	2	-
Kuh sangi	1	1	-
Total	87	82	5

Table 3: Molecular weight of discriminative bands (in kilo base pair) with different species and primers

Species	No.	Size estimated (in kb) of RAPD products with primers			
		AB1-O7	A4	329	327
L.tropica	82	0.9, 1.05	0.7,0.85	2.2	1.7
L.major	5	0.4 to 2.0	0.3 to 2.4	0.2 to 2.0	0.5 to 2.4
L.infantum	*	0.9	0.8, 1.0	0.65, 0.7,0.75	0.95,1.0

* Standard stock

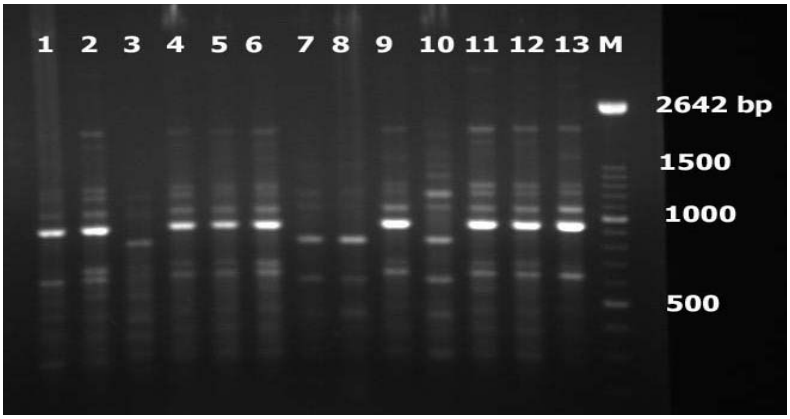

Fig: 1: Random amplified polymorphic DNA (RAPD) profiles obtained from *Leishmania* stocks and isolates with the AB1- O7 primer. Lanes1, 2, 4,5,6,9,11,12,13 represent *L.tropica* and Lanes 3, 7, 8, 10 are *L.major*. Reference stocks Lanes 9 and 10 are: *L.tropica* and *L.major* respectively. M: 100 bp size marker (XIV) (Roche).

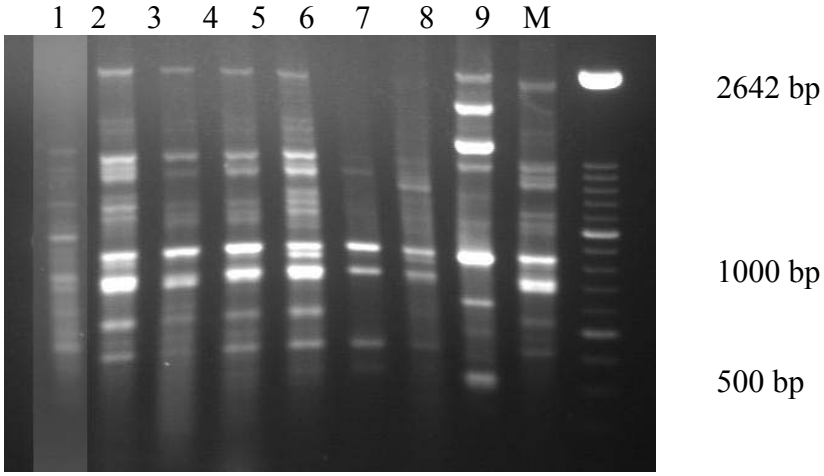

Fig: 2: Random amplified polymorphic DNA (RAPD) profiles obtained from *Leishmani*a stocks and isolates with the A4 primer. Lanes 1, 2 and 8 represent *L.infantum, L.tropica* and *L.major* reference stocks respectively. Lanes 3, 4, 5, 6, 7and 9 are: *L.tropica*. M: 100bp size marker (XIV) (Roche).

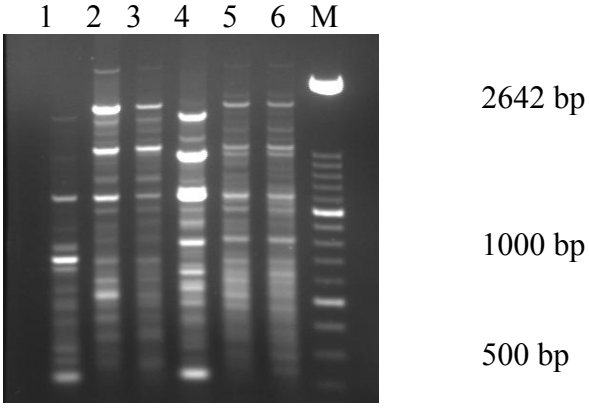

Fig. 3: Random amplified polymorphic DNA (RAPD) profiles obtained from *Leishmania* stocks and isolates with the primer 329. Lanes 1, 4, 5 represent *L.infantum, L. major* and *L.tropica* reference stocks. Lanes 2,3and 6 are: *L.tropica.* M: 100 bp size marker (XIV) (Roche).

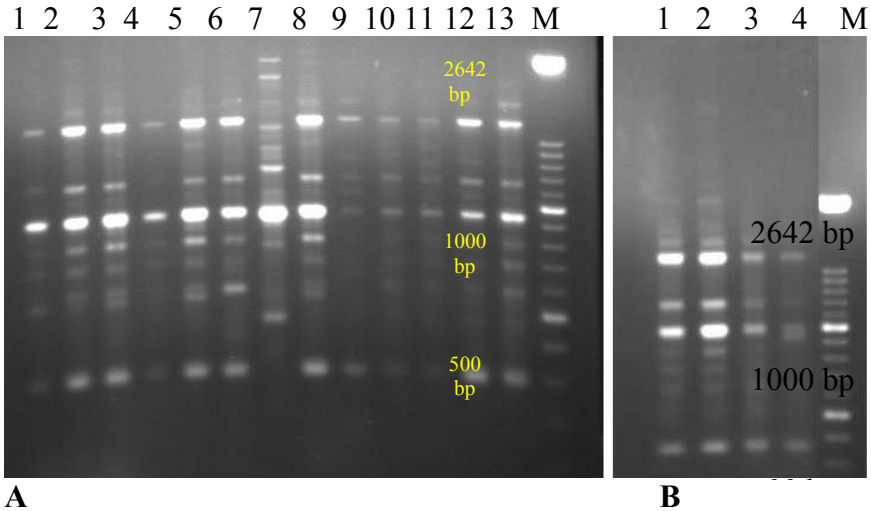

Fig. 4: Random amplified polymorphic DNA (RAPD) profiles obtained from *Leishmania* stocks and isolates with the primer 327. Lanes 7, 8(A) and Lane 4(B) represents *L. major, L.tropica* and *L.infantum* reference stocks respectively. Lanes 1,2,3,4,5,6 and 9,10,11,12,13 (A), and Lane 1,2,3 are *L.tropica;* M, 100bp size marker (XIV) (Roche).

Discussion

Since CL has been the most important endemic disease in Mashhad city (2, 4) and the rate of disease has been increased in various parts of the city in recent years, thus determination of *Leishmania* species seems to be necessary for designing appreciate control programmers. Based on previous experiments (12, 13), we used RAPD-PCR methods for characterization of *Leismania* isolates from Mashhad. RAPD-PCR does not require previous knowledge of primer sequence and only randomly arranged decamer with 60-70% G/C content is sufficient and the technique requires as little as 20ng DNA equivalent to a few parasites per reaction. Likewise, RAPD-PCR can show a genomic diversity among species and which equals between sub-species (22).

Essentially, RAPD technique cannot be applied to clinical samples and is limited to isolated organisms. The results of RAPD are highly dependent on parameters that may vary from one laboratory to another. In some situations, isolation and culture of *Leishmania* are difficult. The best advantage of RAPD-PCR is the simple interpretation of results from PCR-products in gel agarose. These products enable us to discriminate among different species. In the current study we used 87 CL isolates from different parts of Mashhad for identifying by 4 primers selected from a set of 12 primers. As illustrated for the primers AB1-o7, A4, 329 and 327, consistent amplification one or more DNA bands of variety can consider as diagnostic criteria for the identification of *Leishmania* species and different genotype within a species. Moreover, this survey showed that these primers could be used to distinguish the isolates up to different genotype level but AB1-07 primer is more specific and could be used to obtain discriminative bands among different species of *L.tropica, L.major* and *L.infantum.* Several studies have showen that differences between the agents of CL in old world (*L.major & L.tropica)* may be related to different factors, such as morphological and biological characteristics of the parasite (1). In our experiments with inbred mice (Balb/C), *L.major* could produce ulcer at the base of tail of 4 out of 5 animals, 4 - 12 weeks post inoculation. Interestingly, all the isolates were already determined as *L.major* by RAPD-PCR technique. Therefore, a strong correlation is observed between RAPD and animal inoculation. Previous studies showed that subcutaneous injection of *L.major* could produce active lesion in inbred and outbred small white mice but *L.tropica* could not produce any lesion in the same animals even with a large inocolum size (23).

In conclusion, characterization of *Leishmania* isolates collected from different parts of Mashhad city showed that *L.tropica* is predominant agents of CL and is distributed in the most endemic areas of the city. Moreover, this study revealed that RAPD method in spite of some drawbacks is a suitable and powerful tool for characterization of *Leishmania* species.

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