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Article *in* Acta Tropica · May 2006

DOI: 10.1016/j.actatropica.2006.01.010 · Source: PubMed

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Leishmania major: Genetic heterogeneity of Iranian isolates by single-strand conformation polymorphism and sequence analysis of ribosomal DNA internal transcribed spacer

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Received 1 September 2005; received in revised form 15 January 2006; accepted 26 January 2006
Available online 2 March 2006

Abstract

Protozoan parasites of *Leishmania major* are the causative agents of cutaneous leishmaniasis in different parts of Iran. We applied PCR-based methods to analyze *L. major* parasites isolated from patients with active lesions from different geographic areas in Iran in order to understand DNA polymorphisms within *L. major* species. Twenty-four isolates were identified as *L. major* by RFLP analysis of the ribosomal internal transcribed spacer 1 (ITS1) amplicons. These isolates were further studied by single-strand conformation polymorphism (SSCP) analysis and sequencing of ITS1 and ITS2. Data obtained from SSCP analysis of the ITS1 and ITS2 loci revealed three and four different patterns among all studied samples, respectively. Sequencing of ITS1 and ITS2 confirmed the results of SSCP analysis and showed the potential of the PCR-SSCP method for assessing genetic heterogeneity within *L. major*. Different patterns in ITS1 were due to substitution of one nucleotide, whereas in ITS2 the changes were defined by variation in the number of repeats in two polymorphic microsatellites. In total five genotypic groups LmA, LmB, LmC, LmD and LmE were identified among *L. major* isolates. The most frequent genotype, LmA, was detected in isolates collected from different endemic areas of cutaneous leishmaniasis in Iran. Genotypes LmC, LmD and LmE were found only in the new focus of CL in Damghan (Semnan province) and LmB was identified exclusively among isolates of Kashan focus (Isfahan province). The distribution of genetic polymorphisms suggests the existence of distinct endemic regions of *L. major* in Iran.

Keywords: *Leishmania major*; Genetic analysis; ITS1-RFLP; PCR-SSCP; Internal transcribed spacer

1. Introduction

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite *Leishmania.* It is a serious public health problem and affects over 12 million people in many parts of the

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doi:10.1016/j.actatropica.2006.01.010

world (WHO, 1990). The disease is prevalent in many areas of Iran in which *Leishmania major* and *L. tropica* are the primary agents of zoonotic cutaneous leishmaniasis (ZCL) and anthroponotic cutaneous leishmaniasis (ACL), respectively (Nadim and Seyedi-Rashti, 1971). Hyperendemic foci of ZCL have been reported in the north, north-eastern, west, south-western, and central parts of Iran (Nadim and Faghih, 1968; Javadian and Mesghali, 1974; Javadian et al., 1976; Alimohammadian et al., 1999; Tashakori et al., 2003). ACL is endemic in many large cities including Tehran, Shiraz, Mashhad, Kerman, and small cities such as Bam (Seyedi-Rashti and Nadim, 1967; Nadim et al., 1969; Nadim and Aflatonian, 1995; Seyedi-Rashti et al., 1984; Moaddeb et al., 1993; Sharifi et al., 1997). *Leishmania* organisms have been classified as different species primarily on the basis of clinical, biological and epidemiological criteria (Chance, 1979). At present, the standard method for identification of *Leishmania* isolates is based on isoenzyme typing (Rioux et al., 1990); however, this method is slow, laborious, expensive and requires estimation of the profiles of 15 different enzymes. To overcome such difficulties, numerous DNA based methods have been developed in the last decade to evaluate genetic diversity within *Leishmania* species and strains. These assays target the amplification of kinetoplast DNA, rDNA, repetitive nuclear DNA, mini-exon genes and microsatellite DNA sequences (Jackson et al., 1984; Ramirez and Guevara, 1987; Cupolillo et al., 1995; Ramos et al., 1996; Piarroux et al., 1993; Bulle et al., 2002; Marfurt et al., 2003a; Schönian et al., 2003). High levels of inter and intra species variation have been observed in Old and New World *Leishmania* species in the ribosomal DNA internal transcribed spacers (ITS1/ ITS2) (Cupolillo et al., 1995, 2003; Schönian et al., 2000, 2001a,b; El Tai et al., 2000, 2001; Berzunza-Cruz et al., 2002). Here, we applied ITS1-RFLP as a tool for identification of *Leishmania* species. For a further characterization of DNA polymorphisms within *L. major* isolates from different parts of the country, we used single-strand conformation polymorphism analysis (SSCP) of the amplified ITS1 and ITS2 regions and DNA sequencing of representative strains of each SSCP pattern.

2. Material and methods

2.1. Parasites

Twenty-four isolates from skin lesions of Iranian patients with cutaneous leishmaniasis were examined. The patients were selected randomly from typical ZCL foci including Kashan (Isfahan province) and Tehran in the center, Dezful (Khuzestan province) in the south-western region, Dehloran (Ilam province) in the west and Damghan (Semnan province) in the north (Fig. 1). An outline of the geographic distribution of the isolates is shown in Table 1. Two reference strains were used in the study: the Iranian reference strain of *L. major* (MRHO/IR/75/ER), kindly provided by Dr. Javadian, School of Public Health, Tehran University of Medical Sciences, and the *L. major* genome sequencing reference strain (MHOM/IL/80/Friedlin).

2.2. DNA preparation

DNA from cultured *Leishmania* strains was extracted as described by Kelly (1993). Briefly, parasites from a 15 ml mid-logarithmic phase of bulk culture were harvested by centrifugation (700 × *g* for 20 min at 4 °C) and washed three times in ice-cold sterile PBS (pH 7.2). The pellet was resuspended in 1 ml sterile cell lysis buffer (125 mM NaCl, 125 mM EDTA, 2.5% w/v sodium dodecyl sulfate (SDS), 125 mM Tris, pH 8.0) with 100 μg/ml proteinase K and incubated at 56 °C for 3 h. The DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Finally, the DNA was dissolved in 200 μl distilled water and stored at 4 °C. DNA concentration was measured spectrophotometrically.

2.3. PCR amplification of ITS1 and ITS2

PCR was used to amplify the rDNA ITS1 region, which separates the genes coding for the SSU rRNA and 5.8S rRNA, using the primers L5.8S (5′-TGATACCACTTATCGCACTT-3′)/ LITSR (5′-CTGGATCATTTTCCGATG-3′). The ITS2 region, which separates the genes coding for the 5.8S rRNA and LSU rRNA, was amplified with primers L5.8SR (5′-AAGTGCGATAAGTGGTA-3′)/ LITSV (5′-ACACTCAGGTCGTAAAC-3′). PCR conditions have been described by El Tai et al. (2001) and Schönian et al. (2001a). PCR products were separated in a 1% agarose gel at 100 V in 1× TBE buffer and visualized under ultraviolet light after staining in a 0.5 μg/ml solution of ethidium bromide for 15 min.

2.4. Restriction analysis of amplified ITS1

ITS1 PCR products (10 μl) were digested with *HaeIII* for 2 h at 37 °C, using the conditions recommended by the manufacturer (Hybaid GmbH Heidelberg, Germany). Restriction fragments were separated in 2% MetaPhor agarose gels (FMC BioProducts Rockland,

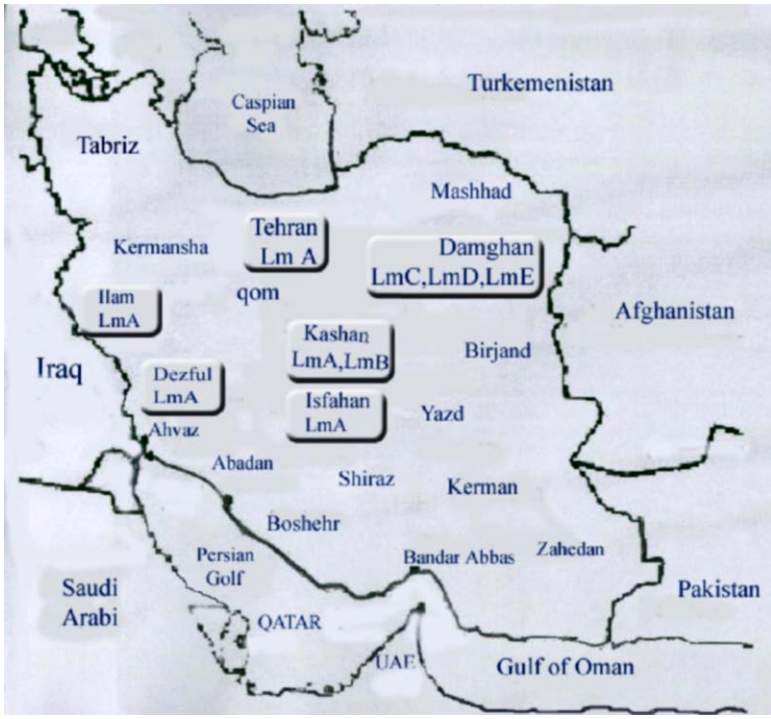

Fig. 1. Leishmaniasis-endemic areas of Iran from which all isolates included in the study were obtained and distribution of different genotype in each studied area.

Table 1
Strains of *Leishmania major* analyzed in this study and results of DNA typing

WHO code	Origin	ITS1-SSCP pattern	ITS2-SSCP pattern	Identified genotyper according PCR-SSCP
MHOM/IR/00/PII 1	Kashan, Iran	A1	A2	Lm A
MHOM/IR/00/PII 2	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 3	Tehran, Iran	A1	A2	Lm A
MHOM/IR/00/PII 4	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 5	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 6	Kashan, Iran	A1	A2	Lm A
MHOM/IR/00/PII 7	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 8	Kashan, Iran	A1	A2	Lm A
MHOM/IR/00/PII 9	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 10	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 11	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 12	Kashan, Iran	B1	B2	Lm B
MHOM/IR/00/PII 13	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 14	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 15	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 16	Dehloran, Iran	A1	A2	Lm A
MHOM/IR/00/PII 17	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 18	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 19	Dezful, Iran	A1	A2	Lm A
MHOM/IR/00/PII 20	Dehloran, Iran	A1	A2	Lm A
MHOM/IR/00/PII 21	Damghan, Iran	C1[a]	D2[a]	Lm E
MHOM/IR/00/PII 22	Damghan, Iran	B1	D2[a]	Lm D
MHOM/IR/00/PII 23	Damghan, Iran	C1[a]	C2[a]	Lm C
MHOM/IR/00/PII 24	Damghan, Iran	B1	D2[a]	Lm D
MRHO/IR/75/ER	Isfahan, Iran	A1	A2	Lm A

[a] Genotypes with two sequences.

ME, USA) in 1× TBE buffer and visualized by staining with ethidium bromide.

2.5. SSCP analysis

ITS1 and ITS2 fragments were screened for polymorphisms by single-strand conformation analysis as described by El Tai et al. (2001).

2.6. ITS sequencing

PCR products of isolates which displayed different profiles in SSCP analysis were directly sequenced. Prior to sequencing the PCR fragments were purified using QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) according to the manufacturer's protocol. Sequencing reactions were carried out with a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) using the PCR conditions recommended by the manufacturer. Samples were then analysed in a 6% polyacrylamide gel on an automated sequencer A373 (Applied Biosystems). Each region was sequenced using the same primers as for the PCR.

3. Results

PCR amplification of ITS1 and ITS2 from all *Leishmania* isolates yielded fragments of about 340 bp and 700 bp, respectively. No variability in PCR product size was observed within the isolates studied using agarose gels. All samples were identified as *L. major* by digestion of ITS1 PCR products with *HaeIII* (data not shown). SSCP analysis of the ITS1 region revealed three polymorphic profiles among all 24 samples examined. These different patterns were coded as A1, B1 and C1 (Fig. 2). Two different profiles were detected in isolates from the Kashan (A1 and B1) and Damghan (C1 and B1) provinces, but only one pattern (A1) was identified in samples collected from Dehloran (Ilam) and Dezful (Khuzestan). ITS2-SSCP analysis revealed four different patterns in all tested DNA samples which were designated as A2, B2, C2 and D2 (Fig. 3). Again, two different profiles (A2 and B2) were detected in isolates from Kashan. Isolates from Dezful and Dehloran displayed only one ITS2 pattern (A2), similarly as in ITS1. Two isolates from Damghan (PII 22 and PII 24) had the profile D2 instead of B2, despite having an ITS1 profile (B1) similar to those of Kashan. Strains PII 23 and PII 21 showed Damghan specific profiles C1/C2 and C1/D2, respectively. In summary, five different ITS genotypes (ITS1 and ITS2) were found among all strains of *L. major* tested. They were named as LmA, LmB, LmC, LmD, and LmE (Table 1). The LmA genotype was detected in 12 (50%) of the 24 isolates collected from different endemic areas of CL in Iran including Kashan, Dehloran, Dezful and Tehran. Genotypes LmC, LmD and LmE were found only in the new focus of CL in Damghan and LmB

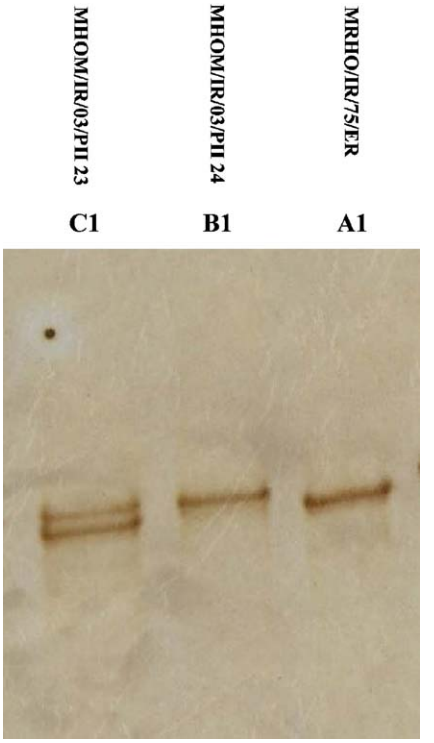

Fig. 2. Example of SSCP profiles generated by ITS1. Each SSCP profile is designed by a capital letter.

Fig. 3. Example of SSCP profiles generated by ITS1. Each SSCP pattern is marked using capital letters.

was identified exclusively among isolates of the Kashan focus (Table 1).

Sequencing of ITS1 confirmed and explained the SSCP profiles of the strains examined. SSCP profiles A1 and B1 differed only in one single nucleotide position (113: G or A, Fig. 4A) (GeneBank accession nos. AY550178 and AY573187), whereas profile C1 displayed both sequence variants at one time. Sequence analysis of ITS2 revealed two sequence types (Fig. 4B), defined by variation in the number of repeats in two polymorphic microsatellites. SSCP profile A2 (GeneBank accession no. AJ786163) was characterized by $(AT)_8$ and $(TA)_4(TG)(TA)_5$, whereas profile B2 (GeneBank accession no. AJ786165) had $(AT)_7$ and $(TA)_4(TG)$, respectively at the same loci. Sequencing of strain PII 23, the single strain with SSCP profile C2, revealed the presence of two distinct sequences. Because differences in the two sequences are based on the numbers of microsatellite repeats, it was impossible to separate them without cloning. SSCP profile D2 (strains PII21 and PII 22) also had a double sequence, with the sequence of SSCP profile B2 being dominant.

4. Discussion

In the past decade DNA-based techniques have improved the identification of *Leishmania* parasites at species and strain levels. In this study, we applied ITS1-RFLP for species identification and SSCP analysis to detect genetic diversity within *L. major*. This technique has been described to detect single base differences in genes (Gasser, 1997). It is relatively quick and simple to perform and requires a relatively small number of parasites. To our knowledge, this work is the first application of PCR-linked SSCP to screen for genetic variation within *L. major* and the results can be summarized as follows: (1) all isolates were identified as *L. major* by ITS1-RFLP; (2) DNA polymorphism within *L. major* isolates was detected in both ITS1 and ITS2 regions by SSCP analysis; (3) DNA sequencing of ITS1 and ITS2 confirmed the results of SSCP analysis for genotypes LmA and LmB. Strains PII 21 and PII 23 both showed two simultaneous ITS1 sequence variants, confirming the SSCP profile, where two bands (A1 and B1) were present. The same phenomenon was observed for ITS2 sequences of PII 21, PII 22 and PII 23. This can be due to either strain heterozygosity or mixed strains, as isolates were not cloned. A third possible explanation is the sequence heterogeneity among different copies of the whole rDNA locus, as suggested previously for *L. tropica* (Schönian et al., 2001a). However, the two simultaneous sequences were found in all

(A)

Genotypic groups	Strain	Region	Accession number	position (110-120)
LmA	MHOM/IR/00/PII1	ITS1	AY550178	TC**G**AAATTTG
Lm B	MHOM/IR/00/PII2	ITS1	AY573187	TC**A**AAATTTG

(B)

Genotypic group	Strain	Region	Accession number	position (237-260)	position (627-648)
LmA	MHOM/IR/00/PII1	ITS2	AJ786163	ATATATATATATATA**T**GTGTGT	TATATATATGTATA TATATATG
Lm B	MHOM/IR/00/PII2	ITS2	AJ786165	ATATATATATATAT. . GTGTGT	TATATATAT. . . . . .G

Fig. 4. Alignment of the variable parts of the ITS1 (A) and ITS2 (B) sequences of the different SSCP profiles detected within *L. major* isolates.

isolates from a single focus but not in Kashan, despite this focus having both LmA and LmB genotypes circulating and the largest number of isolates, which make mixed infections unlikely. We have demonstrated the presence of *L. major* in the studied endemic areas of Iran, which agrees with previous epidemiological studies (Nadim and Faghih, 1968; Javadian and Mesghali, 1974; Alimohammadian et al., 1999; Tashakori et al., 2003). Polymorphism within ITS has been previously reported within different species of *Leishmania* using molecular methods (Schönian et al., 2000, 2001a,b; Hide et al., 2001; El Tai et al., 2000, 2001; Mauricio et al., 2001; Toledo et al., 2002; Marfurt et al., 2003b). El Tai et al. (2001) detected 11 polymorphic SSCP patterns in 63 *L. donovani* samples from Sudan and Schönian et al. (2001a,b) found 14 SSCP profiles within 29 *L. tropica* strains from different Old World geographical areas. We identified here five different genetic variants. In fact, the degree of diversity found is more similar to *L. donovani* with 11 genotypes for 63 strains and a ratio of 0.17 than to *L. tropica* with 14 genotypes from 29 strains and a ratio of 0.48. None of the patients in this study had traveled to foreign countries 6 months prior to infection. Therefore, the presence of isolates from other countries is ruled out. However, there is no information on travel inside Iran. Our observations indicate extensive distribution of genotype LmA in different endemic foci of CL in Iran, whereas LmB was only found in Kashan and the other related genotypes in Damghan. This may be due to transmission of this genotype via affected soldiers during the Iran–Iraq war from east and south east of Iran to other regions. Indeed, we would expect something similar to happen to the other genotypes. LmA may be the most ancient genotype (and the others evolved from it) or the most successful in terms of adaptation to new foci. On the other hand, the genotypes found at Damghan seem to be related to LmB. Unfortunately, most foci were represented by a very small number of strains, so in order to investigate the geographical distribution of genotypes in Iran more samples from different regions of Iran and neighboring countries are required.

In conclusion, the SSCP technique is a powerful diagnostic tool to detect divergence in rDNA ITS regions within species of *Leishmania,* as revealed in previous investigations (El Tai et al., 2000, 2001). Our findings confirmed the effectiveness of this method to detect heterogeneity at the level of a single nucleotide. To date, we have only studied strains of *L. major* isolated from humans, however, for a comprehensive view on the degree of genetic diversity in *L. major*, further investigation which includes more samples from patients, vectors and reservoir hosts from different geographical areas in Iran is required.

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