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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.

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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
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 anthropodemic 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 Meshghali, 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 Affolotan, 1995; Seyedi-Rashti et al., 1984; Mouddeb et al., 1993; Sharifi et al., 1997).

Aftelonian, 1995; Seyedi-Rashti et al., 1984; Moaddeb Rashti and Nadim, 1967; Nadim et al., 1969; Nadim and Seyedi-Rashti, 1971). 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; Schonian 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; Schonian 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 (MHOM/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 L5.8SR (5'-CTGATACCTATCCTTTTG-3') and LITSV (5'-CTGGATCATTT-TCCGATG-3'). The ITS2 region, which separates the genes coding for the 5.8S rRNA and LSU rRNA, was amplified with primers L5.8SR (5'-AAGTGGCA-TAAGTGGTA-3') and LITSV (5'-ACACTCGGTTGCA-3'). PCR conditions have been described by El Tai et al. (2001) and Schonian et al. (2001a). PCR products were separated in a 1% agarose gel at 100V 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>
<thead>
<tr>
<th>WHO code</th>
<th>Origin</th>
<th>ITS1-SSCP pattern</th>
<th>ITS2-SSCP pattern</th>
<th>Identified genotype according PCR-SSCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHOM/IR/00/PII 1</td>
<td>Kashan, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 2</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 3</td>
<td>Tehran, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 4</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 5</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 6</td>
<td>Kashan, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 7</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 8</td>
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<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 9</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 10</td>
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<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 11</td>
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<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 12</td>
<td>Kashan, Iran</td>
<td>B1</td>
<td>B2</td>
<td>Lm B</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 13</td>
<td>Dezful, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 14</td>
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<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
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<td>Dezful, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 16</td>
<td>Dezful, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 17</td>
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<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 18</td>
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<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
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<td>Dezful, Iran</td>
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<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 20</td>
<td>Dehloran, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 21</td>
<td>Damghan, Iran</td>
<td>C1*</td>
<td>D2*</td>
<td>Lm E</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 22</td>
<td>Damghan, Iran</td>
<td>B1</td>
<td>D2*</td>
<td>Lm D</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 23</td>
<td>Damghan, Iran</td>
<td>C1*</td>
<td>C2*</td>
<td>Lm C</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 24</td>
<td>Damghan, Iran</td>
<td>B1</td>
<td>D2*</td>
<td>Lm D</td>
</tr>
<tr>
<td>MHOM/IR/00/PII 25</td>
<td>Isfahan, Iran</td>
<td>A1</td>
<td>A2</td>
<td>Lm A</td>
</tr>
</tbody>
</table>

* 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
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)(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
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 Mezhghali, 1974; Almomahmadian et al., 1999; Tashakori et al., 2003). Polymorphism within ITS has been previously reported within different species of Leishmania using molecular methods (Schonian 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 Schonian 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 for 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 DNA 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 investigations which includes more samples from patients, vectors and reservoir hosts from different geographical areas in Iran is required.

References


