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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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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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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,