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Heterogeneity of the internal transcribed spacer region in *Leishmania tropica* isolates from southern Iran

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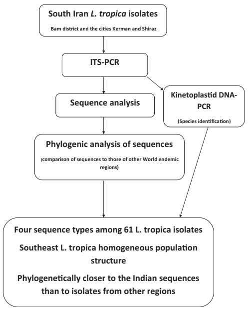
HIGHLIGHTS

- Four haplotypes were identified among the south Iran *L. tropica* isolates.
- All variations occurred in microsatellite regions and were due SNP.
- Iranian *L. tropica* consists of at least two different populations.
- The southeast Iranian *L. tropica* showed a homogeneous population structure.
- The south Iranian population was phylogenetically closer to Indian isolates.

GRAPHICAL ABSTRACT



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ABSTRACT

Most of cutaneous leishmaniasis cases occur in only 7 countries, including Iran. *Leishmania tropica* is the main cause of anthroponotic cutaneous leishmaniasis in Iran. In order to study the heterogeneity and phylogeny of *L. tropica* in southern Iran, a total of 61 isolates were obtained from Bam district and the cities Kerman and Shiraz. The internal transcribed spacer (ITS) from the ribosomal DNA locus was amplified and then analysed by sequencing. Analysis of the ITS sequences showed four haplotypes in the isolates, including 3 haplotypes among the 58 isolates from the south eastern region, including Bam district and Kerman city, and 2 haplotypes among the 3 isolates from Shiraz city. The results showed a monophyletic structure for the south eastern population. In comparison to GenBank sequences of *L. tropica* from different countries, most of the southeast Iranian and Indian isolates are comprised in one cluster, while isolates from other countries and few other Iranian isolates group in a different cluster. Analysis of ITS sequences of south eastern *L. tropica* showed a homogeneous population which could be the basis for other molecular epidemiology studies using more discriminative markers and tracing possible changes in the population structure of *L. tropica*.

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1. Introduction

Leishmania tropica is commonly known as the causative agent of anthroponotic cutaneous leishmaniasis (ACL) in the Old World; however, several studies revealed also zoonotic evidences for *L. tropica* from Africa and the Middle East (Sang et al., 1994; Jacobson et al., 2003; Talmi-Frank et al., 2010). This species was also found to be sporadically involved in causing visceral leishmaniasis (Magill et al., 1993; Sacks et al., 1995).

The vast distribution area of *L. tropica* ranges from Greece (Zakynthos Island), North Africa (Morocco and Tunisia), and new foci in south Sahara (Kenya and Namibia) to the Middle East and Southwest Asia (Turkey, Syria, Iran, Iraq) and it is further reaching to the north-western states Punjab and Rajasthan of India (Jacobson, 2003). *Phlebotomus sergenti* is the most important vector of *L. tropica*, while *Pipistrellus arabicus* and *Phlebotomus guggisbergi* are the main vectors in western Middle East in the northern Galilee focus and in Kenya, respectively (Jacobson et al., 2003; Lawyer et al., 1991). In Iran ACL is found in large or medium-sized cities and their outskirts. It is estimated to be most prevalent in the Bam district of the Kerman province (Sharifi et al., 2012; Razmjou et al., 2009; Sharifi et al., 1998) and Bam city is a well-known focus of ACL. Kerman and Shiraz are large cities of the southern provinces Kerman and Fars, respectively, which are also recognized as important foci of ACL (Sharifi et al., 1998; WHO, 2002). *L. tropica* is the dominant species in Bam district and Kerman city, since almost all CL cases were caused by this species (Sharifi et al., 2012).

Several studies have been performed for a better understanding of population structure and phylogenetic differentiation of *Leishmania* within different species. Such studies provide a basis for tracing epidemiological changes and recognizing pattern of these changes. Most studies in the Old World were focussed on the *Leishmania donovani* complex and *Leishmania major*, while only a few contributed to investigation of polymorphisms of *L. tropica* (Schwenkenbecher et al., 2006; Schwenkenbecher et al., 2004; Schönian et al., 2001; Pratlong et al., 1991). The level of *Leishmania* intra-specific variation has been surveyed by different approaches. The gold standard for *Leishmania* typing is still multilocus enzyme electrophoresis (MLEE) (Rioux et al., 1990), and it has been applied in many different studies (Rioux et al., 1990; Pratlong et al., 1991; Oskam et al., 1998; Nimri et al. 2002). Nevertheless, MLEE needs mass cultivation of parasites and is considered as a time consuming method with restricted discriminatory power (Schönian et al., 2011). Other studies applied different DNA-based approaches to survey *Leishmania* isolates at different taxonomic levels, such as the intra-specific variation. Among these methods are analysis of restriction fragment length polymorphisms (RFLP) and sequencing of various targets (Cupolillo et al., 1995; Noyes et al., 1998; Schönian et al., 2001; Mauricio et al., 2001; Morales et al., 2001; Garcia et al., 2005; Laurent et al., 2007; El-Tai et al., 2001), random amplified polymorphic DNA (RAPD) analysis (Andersen et al., 1996; Zemanová et al., 2004), multilocus sequence typing (MLST) (Mauricio et al., 2006; Zemanová et al., 2007), and multilocus microsatellite typing (MLMT) (Bulle et al., 2002; Schwenkenbecher et al., 2004; Kuhls et al., 2008). Sequence analysis was used in our study because RAPD needs culturing of parasites due to the fact that short, non-specific primers can recognize both host and parasite sequences. Furthermore, the reproducibility of this technique is not reliable between different trials. Moreover, except for kDNA, RFLP has not enough discriminatory power for differentiation of close strains (Morales et al., 2001; Schönian et al., 2011). On the other hand, MLMT and MLST are strongly able to show the variation between the strains, but the methods are not simple to perform (Schönian et al., 2008) and need separate PCR setup for each primer pairs.

The ITS region of ribosomal DNA has been targeted in several studies in order to evaluate the genetic differences of *Leishmania* (El-Tai et al., 2000; Schönian et al., 2000; Schönian et al., 2001; Cupolillo et al., 2003; Kuhls et al., 2005; Almedia et al., 2011). The multi-copy nature of ITS makes it suitable for direct amplification from the clinical samples. Therefore, the present study aims to apply the sequence analysis of the ITS region to evaluate the heterogeneity of *L. tropica* in clinical samples obtained from patients in southern Iran. The main goal of the study is to survey the genetic heterogeneity of *L. tropica* in different regions of the Bam district and to compare it with two other foci of the disease in the south of Iran.

2. Materials and methods

2.1. Samples (strains) and study area

A total of 150 smear preparations were obtained from the patients referring to cutaneous leishmaniasis control centres in Bam and Kerman cities (Kerman province), and Shiraz city (Fars province) (Fig. 1). Because ACL is most prevalent in the Bam district we collected most samples from different regions of this district. The other samples were from the cities Kerman and Shiraz. The samples were taken by scraping the internal border of the skin lesions with a surgical blade, transferred to glass slides and stained by Giemsa stain. The slides were microscopically examined and grouped into five categories according to the numbers of amastigotes (negative, trace, 1+, 2+, and 3+). The 2+ and 3+ slides were selected for further evaluation. Finally, 48, 10, and 3 samples were obtained from Bam district, and the cities Kerman and Shiraz, respectively (Table 1). Thirty-one CL samples were primarily collected in Shiraz, however only few of them were identified as *L. tropica*. The origin of the samples are presented in Table 1 and illustrated in geospatial maps (Fig. 1) which were provided by ESRI- ArcMap 9.3 software from ArcGis software's package (copyright 1999–2008 ESRI Inc).

2.2. DNA extraction

Skin tissue smears were scratched and collected in 1.5 ml microtubes containing lysis buffer (Tris 100 mM, EDTA 10 mM, NaCl 100 mM, SDS 1%, Triton X100 2%). Then, 10 μg/ml Proteinase K was added and the samples were incubated at 56 °C for one hour and then extracted once with phenol/chloroform (25:24 v/v) and once again with chloroform. DNA was precipitated with equal volumes of iso-propanol and also one tenth volume of 3 M NaAc, washed with 70% ethanol, dried, and suspended in 50 μl ultrapure water.

2.3. Kinetoplastid DNA PCR

Kinetoplastid DNA (kDNA) of all the samples was amplified by using the primers 13Z (5′-ACT GGG GGT TGG TGT AAA ATAG-3′) and LiR (5′-TCG CAG AAC GCC CCT-3′) (Noyes et al., 1998) for species identification. The PCR mixture consisted of 12.5 μl of 2× premix (Ampliqon, Denmark), 20 pmol of each primer, 5 μl of template DNA, and water up to 25 μl. The cycling PCR conditions were 95 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 90 s in an Applied Biosystems thermocycler. The PCR products were subjected to 1.2% agarose gel electrophoresis with 0.5 μg/ml ethidium bromide for 90 min at 80 V in TBE buffer and visualized by a transiluminator. A 100 bp DNA ladder was used in each run as size standard.