Natural infection and phylogenetic classification of *Leishmania* spp. infecting *Rhombomys opimus*, a primary reservoir host of zoonotic cutaneous leishmaniasis in northeast Iran

Homa Hajjarana, Mehdi Mohebalia,b,*, Mohammad Reza Abaeic, Mohammad Ali Oshaghic, Zabih Zareid, Sorour Charehdarã, Hamed Mirjalaliã, Meysam Sharifdiniã and Aref Teimouriä

ãDepartment of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; bCenter for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran; cDepartment of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran; dMeshkin-Shahr Research Station, School of Public Health and National Institute of Health Research, Tehran University of Medical Sciences, Tehran, Iran

ãCorresponding author: Present address: School of Public Health, Tehran University of Medical Sciences, P.O. Box: 14155-6446, Tehran, Iran. Tel: + 98 21 88951400; Fax: +98 21 88968258; E-mail: mohebali@tums.ac.ir

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**Background:** In the northeast and central parts of Iran, *Rhombomys opimus* (great gerbil) is the primary reservoir host of zoonotic cutaneous leishmaniasis (ZCL). This study used both parasitological and molecular methods to identify *Leishmania* spp. and their different haplotypes that were circulating in the great gerbil populations in ZCL foci from northeastern Iran.

**Methods:** A cross-sectional study using microscopy, culturing and molecular methods was conducted to detect *Leishmania* parasites in 194 live *R. opimus* in ZCL foci from northeastern Iran during 2010–2011.

**Results:** *Leishmania* spp. were found in 38.1% (74/194) of the samples by microscopy and in 41.2% (80/194) by culturing. Small papules and skin thickening on the upper edge of the ears were observed in 25 (12.9%) of the isolates. PCR-RFLP and PCR direct sequencing of internal transcribed spacer 1 (ITS1) rRNA showed similar infection rates for *L. major* and *L. turanica* in 60 eligible *R. opimus*, only one mixed infection containing both *L. major* and *L. turanica* was found. Phylogenetic analysis was conducted using the ITS1 sequences of 32 isolates that were successfully aligned by comparison of their base sequences with the ITS1 DNA sequence database using ClustalW and MEGA5. The samples were classified into monophyletic clusters (>97% bootstrap). Six haplotypes were observed for *L. major* and seven for *L. turanica*.

**Conclusion:** In northeast Iran, *L. major*, and *L. turanica* naturally circulate in *R. opimus*, and *L. major*/*L. turanica* co-infections also exist. Phylogenetic analysis suggested that *Leishmania* spp. isolated from *R. opimus* are not a monophyletic group.

**Keywords:** *Rhombomys opimus*, *Leishmania major*, *Leishmania turanica*, ITS1 PCR-RFLP, Haplotype, Iran

**Introduction**

The role of different species of gerbils (desert rodents) as reservoir hosts of various *Leishmania* spp. in zoonotic cutaneous leishmaniasis (ZCL) foci had previously been studied.1–3 *Rhombomys opimus*, known as the great gerbil, is the primary reservoir host of *L. major* in arid regions of central Asia, including northeast Iran and Afghanistan.4–5 In the northeast and central parts of Iran, *R. opimus* is the primary reservoir host of ZCL, and there have been some reports about *Meriones lybicus* as a secondary reservoir host.6–8 Moreover, in the west, south and southeast of Iran, *Tatera indica* and *Meriones hurrianae* have been reported as primary reservoir hosts of ZCL.9–11

*R. opimus* makes huge colonies in its territory in the extended deserts of countries such as Turkmenistan, Uzbekistan, Kazakhstan and Tajikistan and can cause important hygiene problems.12,13 There are reports of the existence of two subspecies (*R. opimus* sargadensis and *R. opimus* sodalist) with eight mtDNA haplotypes in Iran that show considerable variations in morphological and ecological characteristics. This suggests that different populations may have different susceptibility to *Leishmania* parasites and different infection rates.7,14

Molecular and iso-enzyme methods for studying the infection of *R. opimus* populations with *Leishmania* parasites have revealed the presence of at least one or a combination of three *Leishmania* parasites (*L. major*, *L. turanica* and *L. gerbilli*) with various infection rates.
under different temporal and spatial situations.\textsuperscript{15} Although it has been shown that \textit{L. turanica} can produce small lesions in BALB/c mice, only \textit{L. major} is a human pathogen; other species are non-pathogenic.\textsuperscript{9,16} In gerbils, \textit{L. turanica} has been the dominant species and normally coexists with \textit{L. major}.\textsuperscript{5,17}

This study used two parasitological and molecular methods to identify \textit{Leishmania} spp. and their different haplotypes that were circulating in the great gerbil populations in ZCL foci from northeastern Iran.

**Materials and methods**

**Study area**

This study was conducted in northeast Iran where an important ZCL focus is located near the border with Turkmenistan. This region covers two main provinces named Golestan and Razavi Khorasan. The climate of the region is generally moderate, with an average annual temperature of 18.2\textdegree C. This survey was conducted during two time periods, July to October 2010 and August to October 2011, in rural areas of Gonbad-e-Qabus and Maraveh Tappeh districts in Golestan province and Sarakhs district in Razavi Khorasan Province (Figure 1).

**Sampling method**

Animals were captured using Sherman\textsuperscript{16} live-traps baited with vegetables, including walnut, cucumber, tomato, and water melon.\textsuperscript{17} The traps were set close to the burrow entrance 2–3 h before sunset and were checked the next morning after sunrise. The captured animals were anaesthetized using 10% ketamine (150 mg/kg), and then transported to the animal facilities of the School of Public Health, Tehran University of Medical Sciences.

Morphological and physical examinations were performed while the animals were anaesthetized.\textsuperscript{18} Their ears were washed and disinfected with detergents and 70% methanol, and impression smears were prepared from the external edges of the ear lobes by cutting small parts with scissors. Four impression slides were prepared for each gerbil, and each rodent was kept in a separate cage in the animal facility for further study.\textsuperscript{6}

**Parasitological examination**

Prepared slides were fixed with absolute methanol and stained with 10% Giemsa (Labtron, Tehran, Iran) and examined under a light microscope (1000x) by an experienced microscopist for detection of the amastigote form of the \textit{Leishmania} parasite. Simultaneously, skin scraping materials from the ears were cultured in RPMI-1640 (Gibco, Life Technologies GmbH, Frankfurt, Germany) supplemented with 10–15% foetal bovine serum (Gibco), 100 IU/ml penicillin and 100 ug/ml streptomycin (Gibco) under aseptic conditions. All media were incubated at 24–25\textdegree C for 4 weeks.\textsuperscript{19}

**Leishmania reference strains**

Reference strains, including \textit{L. major} (JN860745), \textit{L. tropica} (EF653267), \textit{L. infantum} (EU810776) and \textit{L. turanica} (EU395712) were used as positive controls for PCR analysis.

**DNA extraction**

Positive-culture media containing the promastigote form were counted, diluted to a density of $2 \times 10^6$ parasites per ml, harvested, washed twice in sterile PBS (pH: 7.2–7.4) and processed for DNA extraction. DNA was isolated from cultured promastigotes or Giemsa-positive slides using a commercial DNA extraction kit (Roche

![Figure 1. Study areas from northeast Iran where great gerbils (Rhombomys opimus) were collected.](image-url)
Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

PCR-RFLP of internal transcribed spacer 1 rRNA
DNA samples were analysed for internal transcribed spacer 1 (ITS1) using the following primers: LITSR (forward: 5’-CTG GAT CAT TTT CCG ATG-3’) and L5.8S (reverse: 5’-TGA TAC CAC TTA TCG CAC TT-3’). The reaction was conducted using the PCR PreMix (Roche) in a 25-µl total reaction volume. The amplicons, 300–350 bp, were analysed on 1.2% agarose gels and visualized by UV light after staining with GelRed stain. PCR products (10 µl) were digested using FastDigest BsuRI (Fermentas GmbH, Thermo Scientific, St. Leon-Rot, Germany) according to the manufacturer’s instructions. The restriction fragments were analysed by gel electrophoresis on 3% agarose gels (Invitrogen, Life Technologies GmbH, Frankfurt, Germany). Parasite species were determined by comparing the restriction product profiles of the samples with those of the reference species (Figure 2).

ITS1 PCR direct sequencing
We sequenced 32 of the ITS1 PCR products using the LITSR primer. Sequencing was performed by the Sanger method on an ABI 3730 sequencer (Bioneer, Daejeon, South Korea). The resulting sequences were analysed using BLAST and submitted to GenBank. ClustalW alignment and phylogenetic analysis with the construction of a gene tree were performed using the Tamura 3-parameter model in MEGA5. The bootstrap values with 1000 replications were used to assess the reliability of the gene tree.

Ethical approval
All experiments on the great gerbils were performed based on the guidelines of the ethical board of Tehran University of Medical Sciences, Iran.

Results
Parasitological and ITS1 PCR-RFLP
We captured a total of 194 great gerbils (R. opimus), 124 (63.9%) from Golestan Province and 70 (36.1%) from Razavi Khorasan Province (Table 1). Parasitological investigation of 25 symptomatically infected R. opimus showed that 15, 19 and 17 were positive for Leishmania spp. by microscopy, culturing and PCR-RFLP, respectively. Investigation of 169 asymptomatically infected R. opimus showed that 59, 61 and 43 were positive for Leishmania spp. by microscopy, culturing and PCR-RFLP, respectively. There was a significant difference between symptomatically and asymptomatically infected R. opimus in parasitological and molecular results (p < 0.01). Evidence of both L. major and L. turanica was found in symptomatically and asymptomatically infected great gerbils.

ITS1 PCR amplification was performed using DNA extracted from the cultured promastigote forms or Giemsa-positive slides. After amplification, each positive PCR product gave a 300–350-bp band on a 1.5% agarose gel. PCR products from 60 samples and reference species were subjected to BsuRI restriction enzyme digestion. Fragment lengths observed after electrophoresis on a 3% gel were analysed to characterize the Leishmania spp. present in each sample. The digestion revealed two bands for L. major (220, 135 bp), and three bands for L. infantum (200, 80, 60 bp), L. turanica (200, 70, 50 and <50 bp) and L. tropica (200, 60, 50 bp) (Figure 2). Thirty of the isolates (50%) were identified as L. major, while the remaining 30 (50%) were identified as L. turanica. Among the samples, only one displayed mixed natural infection with L. major and L. turanica.

Sequencing results
The PCR products from 32 Leishmania isolates were sequenced using LITSR as the forward primer. Details of the specimens sequenced and submitted to GenBank are shown in Table 2. The L. turanica sequences obtained in this study displayed 99–100% similarity to L. turanica sequences from Mongolia (AJ272380).

Table 1. Distribution of caught Rhombomys opimus in northeast Iran by parasitological and molecular results during 2010–2011

<table>
<thead>
<tr>
<th>Study locations/ provinces</th>
<th>No. of caught rodents</th>
<th>No. R. opimus with dermatological changes</th>
<th>No. positive by microscopy</th>
<th>No. positive by culture</th>
<th>No. positive by PCR-RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golestan</td>
<td>124 (63.9%)</td>
<td>15 (12.1%)</td>
<td>59 (47.6%)</td>
<td>67 (54.0%)</td>
<td>48 (38.7%)</td>
</tr>
<tr>
<td>Razavi–Khorasan</td>
<td>70 (36.1%)</td>
<td>10 (14.3%)</td>
<td>15 (21.4%)</td>
<td>13 (18.6%)</td>
<td>12 (18.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>194</td>
<td>25 (12.9%)</td>
<td>74 (38.1%)</td>
<td>80 (41.2%)</td>
<td>60 (30.9%)</td>
</tr>
</tbody>
</table>

Figure 2. Gel electrophoresis, showing PCR-RFLP results after digestion with the restriction enzyme BsuRI on samples. Lane 1 negative control; Lanes 2 Leishmania major reference; Lane 3 L. major; Lane 4 L. tropica reference; Lane 5 L. turanica; Lane 7 and 8 L. infantum reference; M: molecular weight marker (Roche, 50 bp).
and Turkmenistan (AJ272381). Identical results were obtained when comparing the L. major sequences with GenBank sequences from Iran (EF413077). In the single mixed L. major/L. turanica infection, the species was identified as L. turanica via sequencing (JN860758).

All L. major and L. turanica isolates from this study and those available in GenBank had identical sequences in a special site that could be a useful molecular marker for their identification (Figure 3). This comparison was made using ClustalW (Bioedit software). For example, L. major isolates contained a six-nucleotide deletion at the position 155–160 compared with L. turanica (Figure 3).

In the same manner, all of the L. turanica isolates contained a five-nucleotide deletion at position 238–242 compared with L. gerbili (Figure 3). L. gerbili was found to be very similar to L. turanica. It is also noteworthy that ITS1 sequences of L. gerbili have their own special nucleotide in some parts of the sequence (Figure 3).

Phylogenetic analysis

In this study, we used MEGA5 to determine the similarities among the 32 Leishmania isolates. On the basis of their ITS1 sequences, a Tamura 3-parameter tree (Figure 4) was constructed for all isolates in Table 2.22 In the Tamura tree, the rodent Leishmania strains were grouped into two separate clusters as L. turanica and L. major. Phylogenetic analysis revealed some variations among the rodent strains (13 haplotypes). The L. turanica isolates, with seven haplotypes (H1–H7), formed a clade that was distinct from the clade (6 haplotypes, H8–H13) of L. major isolates (Figure 4).

To study the intra- and interspecies polymorphisms, we used MEGA5 to calculate the distance rate. The intraspecies genetic distance and similarity among a total of 32 sequenced L. major and L. turanica isolates was 0.0159 (98%) and 0.0308 (96%) respectively. The interspecies similarity between L. major and

<table>
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<th>Species</th>
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</table>

Acc no.: Accession number.

a Only 32 out of 80 isolates were submitted to GenBank.
L. turanica was 94%. There was no clear grouping among the 32 isolates according to their geographical origin.

Discussion

Northeast Iran, which is one of the most active ZCL foci, has a long common border with central Asian countries like Turkmenistan, where leishmaniasis in rodents has been well studied. Three Leishmania spp., L. major, L. turanica and L. gerbilli, generally circulate in the R. opimus population of the ZCL foci in this area.\(^{23}\) Here we found that among 60 isolates, the prevalence of L. major (50%) and L. turanica (50%) was equal. No L. gerbilli isolates were observed. In a study conducted by Mirzaei et al. in Turkmen Sahra, northeast of Iran, L. major and L. turanica were identified using molecular methods.\(^{5}\) Preliminary epizootic studies in the rural districts of Esfahan Province, central Iran, showed that in local R. opimus populations, the three Leishmania species mentioned above existed in single species infections or, in some cases, in mixed infections. Evidence has shown that in endemic foci, L. major and L. turanica are the major species circulating among R. opimus, and it appears that the prevalence of L. gerbilli infection is low.\(^{24,25}\)

Akhavan et al.\(^{4}\) showed that the percentage of gerbils infected with L. major, L. turanica and L. gerbilli was 3.2%, 27.4% and 1.1%, respectively, in the central Iran. They found natural mixed infections in wild rodents: L. major and L. turanica (in 15.8%); L. major and L. gerbilli (in 1.1%); and L. major, L. turanica and L. gerbilli (in 2.1%).\(^{26,27}\) We found a single natural mixed infection containing L. major and L. turanica. Akhavan et al. showed that L. turanica was the dominant species in the population of great gerbils in the studied area. Other studies in central Asia and Iran have reported natural infection of sand flies with L. major, L. turanica and L. gerbilli.\(^{26,27}\)

Parasitological examinations (direct smears and cultures) do not differentiate Leishmania spp., and their sensitivity is lower than molecular methods. Molecular methods, such as PCR-RFLP, and sequencing of samples from rodents’ ear sera, microscopy slides or culture samples provide a sensitive and specific alternative to direct methods.\(^{9,28}\) In previous studies, 21 out of 95 Rhombomys samples (22%) were positive by microscopy and 48 (51%) were positive by nested-PCR.\(^{4}\) In our study, microscopy revealed Leishmania spp. in 74 of 194 captured R. opimus (38.1%) to be positive, while culturing and molecular methods revealed Leishmania spp. in 80 (41.2%). Results indicated that molecular methods like PCR-RFLP are sensitive, and specifically distinguish among the
Figure 4. Phylogenetic tree of the ITS1 region nucleotide sequences of Leishmania isolates recovered from Rhombomys opimus in the north east of Iran. The tree was constructed by using the Tamura 3-parameter model in MEGA software version 5. The numbers above the branches indicate the percentage of bootstrap samplings percentages. Branches without numbers have frequencies of less than 70%. Besides the haplotypes reported in this study, (Leishmania turanica H1–H7, L. major H8–H13), a number of accession numbers from other countries were applied in phylogenetic analysis. Gol: Golestan province; RKh: Razavi-Khorasan province; Mon: Mongolia; Uzb: Uzbekistan; Tur: Turkmenistan; IRI: Iran I.R.
three most common Leishmania parasites of \textit{R. opimus}. Leishmania infection in \textit{R. opimus} is very common, but determination of the Leishmania spp. circulating among them is more important. To avoid health issues for people who live in these foci, or travel there, notification of the pathogenicity of \textit{L. major}, \textit{L. turanica} and \textit{L. gerbilli} is important.\textsuperscript{29, 30} It appears that each Leishmania spp. has a relatively limited range of virulence. Only \textit{L. major} is pathogenic for humans, and hence, is very important in the epidemiology of ZCL.\textsuperscript{13, 15} We should also point out the low pathogenicity of \textit{L. turanica} and its epidemiological importance.

According to the Leishmania spp. seasonal distribution, the frequency of \textit{L. turanica} is the highest in warm months (June to August) and that of \textit{L. major} is the highest in cold months (September to December), but the differences were not statistically significant. Studies by Strelkov showed that \textit{L. turanica} is predominant in winter, spring and early summer, while accumulation of \textit{L. major} occurs by the end of the summer (August to September).\textsuperscript{23} Phylogenetic trees derived from the sequences of ITS1 fragments support a clear divergence between \textit{L. major} and \textit{L. turanica}. The haplotype variation detected in the alignment of ITS1 rDNA sequences in both species was relatively high (13 haplotypes; Figure 4). In a phylogenetic study of nine rodent isolates in central Iran, using maximum parsimony analysis, Parvizi et al.\textsuperscript{25} found four haplotypes of \textit{L. major} in a distinct clade. In a separate study, Doudi et al.\textsuperscript{31} found six different genotype groups of \textit{L. tropica} through PCR-RFLP, using an ITS1 marker, in different studied regions.\textsuperscript{1} ITS1 rDNA proved to be conserved amongst populations of a given Leishmania sp. Sequence analyses and distance methods showed that these markers are useful for both intraspecies and interspecies relationships in Leishmania parasites but it seems that ITS1 is unable to resolve strains in a small geographical area. Therefore, there was no correlation between the polymorphisms found among \textit{L. major} and \textit{L. turanica} isolates from different geographical regions within the great gerbil areas. For such studies more rapidly evolving sequences, perhaps from nuclear or kinetoplast DNA markers, are needed.

In conclusion, in northeast Iran, \textit{L. major} and \textit{L. turanica} circulate naturally in \textit{R. opimus} and co-infection is not uncommon. The PCR-RFLP and sequencing methods described in the current study are reliable methods to distinguish Leishmania spp. and determine the different haplotypes of \textit{L. major} and \textit{L. turanica}. Phylogenetic analysis suggested that Leishmania spp. isolated from \textit{R. opimus} are not a monophyletic group. Genetic distance analysis provided further evidence of the occurrence of polymorphisms among Leishmania spp.

**Authors’ contributions:** MM, HH and MRA contributed equally and designed the study. MRA, ZZ and MS gathered the rodent’s samples from the field. MM, HH, MRA, SCH, MS, HM conducted parasitological experiments and Leishmania parasite isolation from the rodents. HH, and AT conducted molecular experiments. MM, HH and MRO analysed the data. HH and HM analysed the phylogenetic data. MM, HH and MAO drafted the manuscript. All authors read and approved the final manuscript.

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