Molecular variation in *Leishmania* parasites from sandflies species of a zoonotic cutaneous leishmaniasis in northeast of Iran

Mitra Sharbatkhori\(^1\)^\(^2\), Adel Spotin\(^3\)^\(^4\), Heshmatollah Taherkhani\(^2\), Mona Roshanghalb\(^3\) & Parviz Parvizi\(^3\)

\(^1\)Laboratory Science Research Centre, Golestan University of Medical Sciences, Gorgan; \(^2\)Department of Parasitology and Mycology, School of Medicine, Golestan University of Medical Sciences, Gorgan; \(^3\)Molecular Systematics Laboratory, Parasitology Department, Pasteur Institute of Iran, Tehran; \(^4\)Parasitology Department, Medical Faculty, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

**Background & objectives:** In the well-known zoonotic cutaneous leishmaniasis (ZCL) focus in Turkmen Sahara, border of Iran and Turkmenistan, ZCL has increased among humans in the past five years. The present study was undertaken to incriminate vectors of ZCL in the region, and to find molecular variation in *Leishmania* parasites.

**Methods:** The sandflies were sampled using CDC light-traps and sticky papers. All the sandflies were identified using morphological characters of the head and abdominal terminalia. DNA was extracted from the dissected thorax and attached anterior abdomen of individual female sandfly. *Leishmania* detection and identification of sandflies were performed using PCR, digestion of *BsuRI* restriction enzyme and sequencing of ITS-rDNA gene and also by semi-nested PCR to amplify minicircle kinetoplast (k) DNA of *Leishmania*.

**Results:** *Leishmania* infections were detected in 26 out of 206 female sandflies. Of the infected sandflies, 18 were *Phlebotomus papatasi* while eight were *P. caucasicus*/*P. mongolensis*. Two infections of *L. turanica* were detected, one in *P. papatasi* and other in *P. caucasicus*/*P. mongolensis* and the rest of the sandflies were found infected with *L. major*.

**Conclusion:** Our finding showed that *L. major* had low diversity with only one common haplotype (GenBank Access No. EF413075). The novel haplotypes were discovered in *L. major* (GenBank Access No. KF152937) and in *L. turanica* (GenBank Access No. EF413079) in low frequency. These *Leishmania* parasites are circulating to maintain infections in the *P. papatasi* and *P. caucasicus*/*P. mongolensis* in Turkmen Sahara.

Key words  Iran; ITS ribosomal DNA; *Leishmania major*; *L. turanica*; *Phlebotomus papatasi*; *P. caucasicus*/*P. mongolensis*

INTRODUCTION

Zoonotic cutaneous leishmaniasis (ZCL) is endemic disease in Iran and *Leishmania major* is the causative agent transmitted by sandflies\(^1\)^\(^2\). Turkmen Sahara district, in northeast of Iran, border of Iran and Turkmenistan, is well-known and one of the most important endemic area of ZCL\(^2\)^\(^3\). The haematophagous females of *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) have been incriminated as the main vector of the parasitic protozoan *L. major* (Yakimoff and Schokhor) (Kinetoplastida: Trypanosomatidae), the principal agent of ZCL\(^4\)^\(^5\)^\(^6\). In some ZCL endemic parts of Iran, as well as *P. papatasi*, other species of phlebotomine sandflies and reservoir hosts, like the gerbil *Rhomomys opimus* (Licht.) (Rodentia: Gerbillidae), have been found infected with *L. major* and unidentified *Leishmania*\(^2\)^\(^5\)^\(^6\). Nevertheless, most *Leishmania* infections have been detected in *P. papatasi* followed by *P. (Paraphlebotomus) caucasicus* Marzinowsky and / or *P. mongolensis* Sinton which are likely to be the main and secondary vectors in Iran\(^5\)^\(^7\).

*Phlebotomus caucasicus* and *P. mongolensis* are frequently found in the burrows of the great gerbil, *R. opimus* and other reservoir hosts in Iran. The females of these sandfly species are similar and can not be separated morphologically and molecularly\(^3\)^\(^8\)^\(^9\).

Our study sites are part of an important ZCL focus in Golestan province, in northeast of Iran (Fig. 1). Recently, according to Disease Control Unit of the Health Centre report, ZCL cases increased significantly and became as an epidemiological problem over the past five years in some locations in Turkmen Sahara\(^10\). The first objective was to determine whether any molecular variation exists in *Leishmania* parasites. The second objective was to develop our knowledge regarding the detection of one or more *Paraphlebotomus* sandflies in which the *Leishmania* parasites survive and continue their transmission cycles. The last, was evaluation and finding new approaches of controlling surveillance of leishmaniasis in Turkmen Sahara focus. In addition, we tried to identify and type molecular *Leishmania* species in *P. papatasi* and *P. caucasicus* as main and potential vectors of ZCL.
in Turkmen Sahara in northeast of Iran. Some of our preliminary data has already been published in Persian in a regional journal.11

The internal transcribed spacers (ITS) and kinetoplast (k) DNA of Leishmania parasites were selected to firmly identify and confirm the molecular analysis using nested and semi-nested PCR, respectively. Also restriction fragment length polymorphism (RFLP) and sequencing of ITS gene were performed for final verification.12–15

MATERIAL & METHODS

**Sandfly collections, identification and extraction of DNA**

Sandflies were sampled from 15 villages of Turkmen Sahara, Golestan province, in northeast of Iran. Villages of our study sites are coordinated in Gonbad-e Qâbus or Gonbad-e Kâvus (37° 15′ 00″ N 55° 10′ 02″ E), Aqqala (37° 00′ 50″ N 54° 27′ 18″ E), and Marveh Tapeh (37° 54′ 15″ N 55° 57′ 21″ E) in a ZCL focus of this region (Fig. 1). Sandflies collected were from animal shelters, gerbil burrows and inside the houses in 2009 and 2010 using CDC light-traps and sticky papers. All the sandflies were identified using morphological characters of the head and abdominal terminalia.17–18 DNA was extracted from the thorax and attached anterior abdomen of each sandfly as previously described. Nested PCR of ITS-rDNA was used for detecting and identifying Leishmania species in sandflies.

**A semi-nested PCR of minicircle kinetoplast (k) DNA for identifying Leishmania species in sandflies**

The primers LINR4 (forward), LIN17 (first-step reverse) and LIN19 (second-step reverse) were used for a semi-nested PCR. The primers anneal within the conserved area of the minicircle and are also based on the Conserved Sequence Blocks recognized by Brewster et al.20

**Restriction fragment length polymorphism (RFLP) of ITS-rDNA gene**

The sequence of standard strain (L. major, MHOM/SU/73/5ASKH; L. infantum, MHOM/TN/80/IPT1; L. turanica, MRHO/MN/83/MNR1 and L. gerbilli, MRHO/UZ/87/KD-87555) which have been registered in GenBank were used for sequencing analysis to select the suitable enzyme for digesting the PCR product in RFLP method.

Leishmania species were analysed using CLC DNA Workbench 5.2 software (CLC bio A/S, Aarhus, Denmark) to select the suitable enzyme which has different cut sites in different species of Leishmania, after sequencing standard strains of it. BsuRI (HaeII) was selected with cut site GG↓CC as an appropriate enzyme for PCR product digestion. Endonuclease digestion was performed in a volume 30 μl include PCR product 10 μl, BsuRI (HaeII) enzyme (Fermentas) 2 μl, 10× buffer 2 μl and distilled water 16 μl for 4 h at 37°C.

Furthermore, standard strains of L. major, L. tropica and L. infantum were used as positive controls. One of two negative controls was without restriction enzyme and the other one had no PCR product. After digesting the PCR product by endonuclease-restriction enzyme using BsuRI (HaeII) enzyme, the fragments were analysed by using electrophoresis on 3% agarose gel containing etidium bromide and ladder 50 bp (Fermentas).

The DNA fragments amplified by PCR were fractionated on 1.5% agarose gels, together with standard DNA fragments to permit sizing. PCR products were directly sequenced to identify Leishmania haplotypes infecting individual female sandflies, and all haplotypes were identified to species by phylogenetic analysis. For this, DNA sequences were edited and aligned using Sequencher© 4.4.1 software, and the multiple alignments of new DNA haplotypes and homologous GenBank sequences were exported into MEGA software for phylogenetic analysis. The homologous GenBank sequences were selected and molecular phylogenetic tree was constructed for the Old World Leishmania species.
### Table 1. *Leishmania* species identified in *P. papatasi* and *P. caucasicus/P. mongolensis* in different villages based on habitats and abdomens position using size of kDNA, RFLP of ITS-rDNA fragment and sequences

<table>
<thead>
<tr>
<th>Location</th>
<th>Data collection</th>
<th>P. papatasi</th>
<th>P. caucasicus/P. mongolensis</th>
<th>RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (+ve)</td>
<td>Abdomen position</td>
<td>Habitat</td>
<td>Total (+ve)</td>
</tr>
<tr>
<td></td>
<td>(ITS) [kDNA]</td>
<td>FF</td>
<td>G</td>
<td>SG</td>
</tr>
<tr>
<td><strong>Gonbade-Kavous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dash boron</td>
<td>70 (12)[12]</td>
<td>0</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Torshakli</td>
<td>24 (2)[2]</td>
<td>0</td>
<td>0</td>
<td>24 (2)</td>
</tr>
<tr>
<td>Shurdgesh</td>
<td>5 (2)[2]</td>
<td>0</td>
<td>0</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Dozoloom</td>
<td>11 (1)[1]</td>
<td>0</td>
<td>0</td>
<td>11 (1)</td>
</tr>
<tr>
<td>Qeshlagh</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Daneshmand</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>Inche boron</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Okhitapeh</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Aqqala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jafar bay</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bibishirvan</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Farghesarepaen</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Marveh Tapah</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gharagol</td>
<td>1 (1)[1]</td>
<td>0</td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Shirjepaeen</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Souzesh</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Kheyrhajecoliya</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>168 (18)[18]</td>
<td>13</td>
<td>28 (1)[87 (17)</td>
<td>3</td>
</tr>
</tbody>
</table>

FF—Full fed; G—Gravid; SG—Semi-gravid; IH—Inside houses; RB—Rhombomys burrows; ASH—Animal shelters.
RESULTS

Sandflies collection, Leishmania infections of sandflies identified by the nested PCR of ITS1-5.8S rRNA gene-ITS2

Sandflies were widespread in September 2009 and 2010. Phlebotomus papatasi was abundant and predominant species in the region. The collections included the putative vectors of L. major causing ZCL in the region and in Iran (Table 1). The females of P. caucasicus and P. mongolensis could not be separated morphologically but, based on the presence of the males, between these two species, most were P. caucasicus8, 21–22. Altogether, 206 female sandflies of two species were collected from 15 villages and screened for Leishmania infections. A total of 26 infections of Leishmania were discovered (18 in P. papatasi and 8 in P. caucasicus/P. mongolensis) (Table 1). For the detection of Leishmania infections, the female sandflies were captured and identified using ITS-5.8S rRNA-ITS2 gene. Two Leishmania infections were unidentified using RFLP. All the 26 independently ITS-rDNA fragments from each species were directly sequenced, to confirm their homology with species-specific GenBank sequences3. Haplotype TSH61 direktly sequenced, to confirm their homology with dently ITS-rDNA fragments from each species wereutations have rarely been screened for Leishmania and few infections have been previously reported20, 26, 30. Sandflies caught in gerbil burrows, contain with most infections needs in the burrows of the reservoir hosts, from where some infected females later disperse to domestic animal shelters8.

Nested PCR of ITS-rDNA have 100% specificity based on sequencing the amplified DNA fragments5, 31. The ITS-rDNA gene was used for phylogenetic analysis of the Leishmania parasites from which the sequences originated based on a previous study in Iran and elsewhere32. The ITS-rDNA sequences obtained from the P. mongolensis/P. caucasicus and P. papatasi showed a similarity to the sequences deposited in GenBank. The results of a detailed phylogenetic analysis including characterization of such an approach could serve as a foundation for understanding the life cycle of Leishmania in sandflies (Fig. 2).

RFLP of (BsuRI [HaeIII] enzyme) ITS-rDNA gene was diagnostic for L. major to compare two mammalian Leishmania (L. turanica and L. gerbilli)13, because of the size of the amplified DNA fragment for L. major (650 bp) compared with three other species of L. donovani, L. infantum and L. tropica (720 bp)16. Semi-nested PCR of minicircle kDNA has the practical advantage of being diagnostic. There is no report for sizing fragment of minicircle kDNA for L. turanica. Based on sequencing the amplified DNA fragments the size of the fragment the visualized obtained bands for L. turanica which was equal to 720 bp similar to the standard strains of L. donovani, L. infantum and L. tropica21.

DISCUSSION

Our findings showed L. major and L. turanica parasite species maintain infections in the P. papatasi and P. caucasicus/P. mongolensis in Turkmen Sahara, but it is important to discover the complementary roles of the sandflies species transmitting each Leishmania species among the reservoir hosts and to people24–26. Out of 26, 24 infections in wild females of P. papatasi and P. caucasicus/P. mongolensis were identified as L. major, and infection rates were significantly higher in P. papatasi from domestic animal shelters (18/18) than in P. papatasi caught in gerbil burrows (0/18) and also inside the houses (0/18). But infection rates were higher for gerbil burrows in P. caucasicus/P. mongolensis (7/8) than in domestic animal shelters (1/8) and no infection was detected in sandflies inside the houses (0/8) (p<0.01). Perhaps P. caucasicus/P. mongolensis prefer to feed blood of rodents and rest in gerbil burrows27–29.

These two habitats are often <300 m apart in the Turkmen Sahara focus of ZCL, but its peridomestic populations have rarely been screened for Leishmania and few infections have been previously reported20, 26, 30. Sandflies caught in gerbil burrows, contain with most infections needs in the burrows of the reservoir hosts, from where some infected females later disperse to domestic animal shelters8.

Leishmania infections of sandflies identified by the semi-nested PCR of minicircle kinetoplast (k) DNA

Most of each minicircle kDNA molecule was amplified by the semi-nested PCR, as reported by Aransay et al15, the size of the fragment was diagnostic for L. major (650 bp) compared with three other Old World species of Leishmania (720 bp). For amplifying minicircle kinetoplast (k) DNA, all the 26 Leishmania positive for ITS-rDNA gene were tried using the semi-nested PCR. Size specificity was 100% for 24 fragments of L. major and two fragments for L. turanica (Table 1).
None of *P. papatasi* and *P. caucasicus/P. mongolensis* in the present study had double infections unlike *R. opimus* in the ex-USSR and also in Iran. In Iran, several studies were carried out and found different *Leishmania* species in various parts of Iran but most of their work was based on conventional methods and some investigators employed molecular methods but they stopped after running and getting band in Agarose gel.30, 32–34.

Regarding our findings in this study, because of limited flight range, sandflies transmission of leishmaniasis within ZCL focus in Turkmen Sahara is often discontinuous, with characteristically small and separate foci close to the reservoir host habitats.29, 35. Also, infection rates were higher in semi-gravid and gravid than in females with red blood meals and for those without blood meals or eggs. Female sandflies need blood for egg production, some of the flies without blood or eggs might have been nulliparous and, therefore, had no chance to become infected.36–37.

The morphological and molecular similarity of female of *P. caucasicus* and *P. mongolensis* prevents a direct investigation of their roles in maintaining transmission of *L. major*9, 24 and other *Leishmania* species of gerbils.5, 7.

Now we can conclude that these sandfly species might be the vectors of *L. major* and *L. turanica* in human and/or in reservoir hosts of ZCL in Turkmen Sahara. In the future, more work should be carried out to test the status of sandflies as vectors of *L. major* and *L. turanica.*

**ACKNOWLEDGEMENTS**

The authors thank Mehdi Baghban for his help in the field work and Elnaz Alaeenovin for help in Molecular Systematics Laboratory. A part of this research was assigned by Mona Roshanghalb, M.Sc. student at Pasteur Institute of Iran, Tehran. The work was supported by the Pasteur Institute of Iran, grant number 367 awarded to Dr Parviz Parvizi. The collections of sandflies were made possible by the assistance of the Centre of Health Service in Gonband and Maraveh Tapeh, Golestan province, Iran. Authors thank Elham Esmaili, for language editing.

**REFERENCES**


7. Parvizi P, Benlarbi M, Ready PD. Mitochondrial and Wolbachia markers for the sandfly *Phlebotomus papatasi*: Little population


Correspondence to: Dr Parviz Parvizi, Molecular Systematics Laboratory, Parasitology Department, Pasteur Institute of Iran, Tehran, Iran. E mail: parp@pasteur.ac.ir; parpparvizi@yahoo.com

Received: 21 August 2013 Accepted in revised form: 17 December 2013