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

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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 *B*suRI 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. turnica* 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]. In some ZCL endemic parts of Iran, as well as *P. papatasi,* other species of phlebotomine sandflies and reservoir hosts, like the gerbil *Rhombomys 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 moleculary *Leishmania* species in *P. papatasi* and *P. caucasicus* as main and potential vectors of ZCL

Fig. 1: Locations of Gonbad-e Kâvus, Aqqala and Marveh Tapeh districts in Turkmen Sahara, Golestan province of Iran, where sandflies were sampled and screened for *Leishmania* infections.

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 Kâvous (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[16] 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[19]. Nested PCR of ITS-rDNA was used for detecting and identifying *Leishmania* species in sandflies.

A total of 206 female sandflies from these collections were screened for infections of *Leishmania* species by nested PCR. The PCR assay was carried out as pre the protocol described by Parvizi and Ready[4]. The first PCR was performed using forward primer IR1 reverse primer IR2. The nested PCR was done using forward primer ITS1F and reverse primer ITS2R4[4].

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[19]. 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. *B*suRI (*H*aeIII) 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, *B*suRI (*H*aeIII) 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 *B*suRI (*H*aeIII) enzyme, the fragments were analysed by using electrophoresis on 3% agarose gel containing ethidium 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 phylogenetc 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

Data collection			*P. papatasi*							*P. caucasicus/P. mongolensis*						RFLP			
Location		Total (+)ve	Abdomen position			Habitat			Total (+)ve	Abdomen position			Habitat			*P. papatasi*		*P. caucasicus/ P. mongolensis*	
Districts	Villages	(ITS) [kDNA]	FF	G	SG	IH	RB	ASH	(ITS) [kDNA]	FF	G	SG	IH	RB	ASH	*L. major*	*L. turanica*	*L. major*	*L. turanica*
Gonbade-Kavous	Dash boron	70 (12) [12]	13	10	47 (12)	0	7	63 (12)	6 (2)[2]	0	3 (2)	3	1	3 (2)	2	11	1	2	0
	Torshakli	24 (2) [2]	0	0	24 (2)	0	0	24 (2)	4 (2) [2]	0	1	3 (2)	0	1 (1)	3 (1)	2	0	1	1
	Shurdgesh	5 (2) [2]	0	0	5 (2)	0	0	5 (2)	1	0	1	0	0	1	0	2	0	0	0
	Dozoloom	11 (1) [1]	0	0	11 (1)	0	0	11 (1)	2	0	2	0	1	1	0	1	0	0	0
	Qeshlagh	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Daneshmand	20	0	1	19	0	1	19	0	0	0	0	0	0	0	0	0	0	0
	Inche boron	3	0	2	1	1	2	0	2	0	2	0	2	0	0	0	0	0	0
	Okhitapeh	5	0	1	4	0	5	0	0	0	0	0	0	0	0	0	0	0	0
Aqqala	Jafar bay	2	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
	Bibishirvan	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Farghesarepaeen	8	0	0	8	0	0	1	2	0	0	2	0	1	1	0	0	0	0
Marveh Tapeh	Gharagol	1 (1)[1]	0	1 (1)	0	0	6	3 (1)	10 (4)[4]	3 (1)	6 (3)	1	0	9 (4)	1	1	0	4	0
	Shorjepaeen	3	0	0	3	0	0	8	1	0	3	1	0	4	0	0	0	0	0
	Souzesh	2	0	0	2	1	1	0	6	2	2	2	2	4	0	0	0	0	0
	Kheyrkhajeoliya	2	0	0	2	1	1	0	1	0	1	0	0	1	0	0	0	0	0
	Total	168 (18) [18]	13	28 (1)	87 (17)	3	26	135 (18)	38 (8)[8]	5 (1)	21 (5)	12 (2)	6	25 (7)	7 (1)	17	1	7	1

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 ITS1-5.8 S 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. caucasicus*[8, 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 sequences[3]. Haplotype TSH61 (GenBank Access No. EF413075) was identified in some GenBank sequences from strains of *L. major* originating from different locations including Iran, and it predominated in Iranian sandflies infected with this species. New haplotype TSH94 (GenBank Access No. KF152937) of *L. major* was also found in three infections.

Two infections of *L. turanica* were detected in one *P. papatasi* and other in *P. caucasicus*/*P. mongolensis* (Table 1). Both single *P. papatasi* and *P. caucasicus P. mongolensis* sandflies with *L. turanica* sequences had the same haplotype TSH91 (GenBank Access No. EF413079). Haplotype TSH91 had previously been isolated from *R. opimus* from Mongolia and some Republics of the ex-USSR and also in Iran[23].

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 al*[15], 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).

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 people[24–26]. 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 burrows[27–29].

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 reported[20, 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 shelters[8].

Nested PCR of ITS-rDNA have 100% specificity based on sequencing the amplified DNA fragments[5, 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 elsewhere[32]. 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 (*B*suRI [*H*aeIII] 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. tropica*[21].

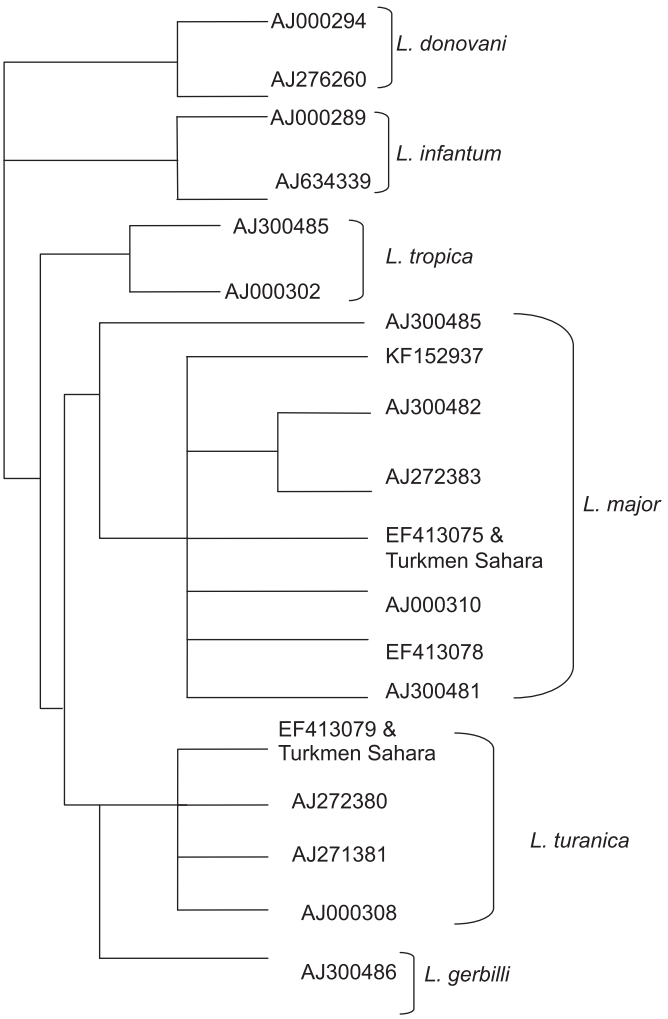

Fig. 2: Unrooted neighbour-joining tree showing the relationships of the haplotypes of the ITS1-5.8SrRNA gene fragment for the isolates of *Leishmania* species using PAUP.

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.*

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