Original Article

Molecular Characterization of *Leishmania* Infection from Naturally Infected Sand Flies Caught in a Focus of Cutaneous Leishmaniasis (Eastern Iran)

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(Received 15 Feb 2012; accepted 16 March 2013)

Abstract

Background: Cutaneous leishmaniasis due to *Leishmania major* is a serious and increasing problem affecting many rural areas of 17 out of 31 provinces in Iran. Little is known about sand fly fauna and leishmaniases in Eastern Iran and no study has been carried out in Sarbisheh County. The aim of this study was to determine sand flies composition and probable *Leishmania* infection to find the probable vectors of leishmaniasis in Sarbisheh district.
Methods: Sand flies were caught using both sticky papers and CDC light traps in August 2010. They were identified morphologically and analyzed for *Leishmania* infection by amplification of ITS-rDNA.
Results: Totally, 842 specimens were caught and 8 species recorded. They belonged to the genera *Phlebotomus* and *Sergentomyia*: *P*. (*Phlebotomus*) *papatasi*, *P.* (*Paraphlebotomus*) *sergenti*, *P*. (*Pa.*) *caucasicus*, *P*. (*Pa.*) *mongolensis*, *P*. (*Pa.*) *jacusieli*, *S*. (*Sergentomyia*) *dentata*, *S.* (*Se.*) *sintoni* and *S.* (*Sintonius*) *clydei.* All collected females were processed for *Leishmania* DNA detection by PCR amplifying of Internal Transcribed Spacer1 (partial sequence), 5.8S (complete sequence) and ITS2 (partial sequence) fragments. Thirteen females were positive for *Leishmania* DNA. The sequencing of the 430 bp amplicons indicated that 9 *P. papatasi* and 3 females belonging to the Caucasicus group carried *L. major* DNA whereas one *P. sergenti* carried *L. tropica* DNA.
Conclusion: *Phlebotomus papatasi* and *P. sergenti* are, like in several places, the probable vectors of cutaneous leishmaniases in this emerging or unknown focus of cutaneous leishmaniases.

Keywords: *Leishmania major*, *Leishmania tropica*, ITS-ribosomal DNA, Iranian Sand fly

Introduction

Cutaneous Leishmaniases (CL) due to *Leishmania major* Yakimoff and Schokhor, 1914 and *L. tropica* Wright, 1903 (Kinetoplastida: Trypanosomatidae) occur serious increasing health problems in Iran, affecting mainly rural areas in 17 out of 31 provinces in Iran (Yaghoobi-Ershadi 2012). The most important foci are due to *L. major*. They are endemic and are located in Turkemen Sahara and Lotf Abad in north east of Iran, Abardejh, Esfahan and Yazd districts in center of Iran, Fars and Sistan v Baluchestan provinces in south and south east, Ilam and Khuzestan provinces in south west of the country (Nadim and Seyedi-Rashti 1971, Javadian et al. 1998, Yaghoobi-Ershadi et al. 2005, Nekouie et al. 2006, Oshaghi et al. 2010).

In Iran, as in many foci located all over the world, the main vector of *L. major* is *P. papatasi* Scopoli, 1786 (Nadim and Seyedi-Rashti 1971, Yaghoobi-Ershadi et al. 2005, Parvizi and Ready 2008). However, other species belonging to the subgenus *Phlebotomus* or females belonging to the Caucasicus group have also been reported as vectors of *L. major*, the latter especially as secondary vectors in areas where *P. papatasi* is not recorded, or at low densities (Nadim and

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Published Online: August 31, 2013

Seyedi-Rashti 1971, Yaghoobi-Ershadi et al. 1994). *L. tropica* is mainly transmitted by *P. sergenti* Parrot, 1917 (Oshaghi et al. 2010).

Despite the report of several new foci of cutaneous leishmaniases in Iran and the potential spread of the disease in the country according to the Iranian authorities, leishmaniases were unknown in the province of Khorasan-e-Jonoubi till now. According to the report of the Ministry of Health, 417 new cases of CL were reported from Khorasan-e-Jonoubi in 2009. An increase of 4% was recorded in 2010 compared to 2009 (Iran Ministry of Health and Medical Education 2010).

In the present study, Phlebotomine sand flies were sampled in Sarbisheh County bordering Afghanistan (Fig. 1). According to our knowledge, no entomological or epidemiological study has been carried out in this region. The aim of this study was to carry out a pilot study on the sand fly species composition in the prospected area and to have a look on their *Leishmania* infection.

Materials and Methods

Phlebotomine sand flies sampling was carried out from 18th to 27th August, 2010, in Sarbisheh county in East of Iran, 58˚48' N and 32˚34' E, at an altitude of about 1800 meters above sea level (Fig. 1). The climate in this area bordering Afghanistan is mild and dry. The highest temperature in summer, 40 degrees above zero and the lowest in winter, 23 degrees below zero. The normal annual rainfall is 228 mm in Sarbisheh County. During the collection period, the average minimum and maximum temperatures were 31˚C and 39 ˚C while the mean humidity was 54%.

In this cross-sectional study, sampling was done in rural regions of Sarbisheh County during 10 summer nights. We selected two catching methods: sticky papers and CDC miniature light traps. The goal of our study was not to collect data of the relative abundance of the species, that could be done by using sticky papers, but to mix two trapping methods in order to maximize the opportunity to catch all the species from a prospected location, photophilic or not.

Sticky papers consist of white sheets 21x 29.7 cm coated with castor oil placed in different habitats and various biotopes: indoors, animal shelters, and outdoors (scuppers, wall cracks, burrows, and vegetation). They were put on the ground with a stick, or rolled into cones and placed in the interstices of stone walls, in walls made of clay, or placed vertically in cracks, crevices and large boulders. CDC miniature light traps were put in the same locations. All these traps were installed before sunset and remained functional throughout the night until the next morning. Sand flies specimens were stored in 96% ethanol and kept in refrigerator (-20 ˚C) for further analysis.

After recording the sampling data and locations, sand fly specimens were washed in 1% detergent then in sterile distilled water. Each specimen was then dissected in fresh drop of sterile normal saline by cutting off the head and genitalia with sterilized entomological needles, then were mounted in Berlese medium and identified using the identification key of Theodor and Mesghali (1964). We considered the females of *P. caucasicus* Marzinovsky, 1917 and *P. mongolensis* Sinton, 1928 indistinguishable (Artemiev and Neronov 1984, Parvizi et al. 2010). All these females were identified as "Caucasicus group". Thorax, abdomen, legs and wings were stored in the sterile 1.5 ml microtube, then frozen and defrosted twice to break up tissue using a sampler tips or pestle, with grinding mix. Then SDS mix was used to denature proteins associated with the DNA, and then ice cold 8 M potassium acetate was added to effectively remove the SDS-bound proteins from solution. Cell debris and proteins were separated from the DNA by centrifugation and the DNA in

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Published Online: August 31, 2013

the supernatant was precipitated over night at -20 ˚C in 96% ethanol. Following ethanol precipitation, the DNA was dissolved in 1X-TE (10 mM Tris–HCl, 1 mM EDTA PH 8.0) and stored at 4 ˚C.

Leishmania DNA was detected in Phlebotomine sand flies by amplification of the first Internal Transcribed Spacer of the ribosomal DNA (partial sequence), 5.8S ribosomal RNA gene (complete sequence) and Internal Transcribed Spacer 2 (partial sequence) that able to detect *L. major* and *L. tropica*. This fragment was amplified using the forward (ITS_1F) and reverse (ITS_2R_4) primers (Parvizi and Ready 2008). The length of PCR band was 430 bp for *L. major* and *L. tropica*. Double distilled water and DNA from *L. major* and *L. tropica* were used as negative and positive controls for each batch of PCR.

Standard PCR was performed in a 45 µl volume using extracted DNA solution 5 µl, 10X buffer 4 µl, MgCl2 2.4 µl, dNTPs 4 µl, *Taq* polymerase 0.4 µl, DDW 28.6 µl and 0.3 µl from each forward (ITS1F: 5′-GCAGCTGGATCATTTTCC-3′) and reverse (ITS2R4: 5′-ATATGCAGAAGAGAGGAGGC-3′) primers and according to the PCR Thermocycler program for *Leishmania* parasite (Parvizi and Ready 2008). Amplicons were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide.

PCR products were sequenced both directions directly using the ITS_1F and ITS_2R_4 primers which used for DNA amplification.

Sequences compared to homologous sequences in GenBank thanks to the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST: www.ncbi.nlm.nih.gov/BLAST). Strains were considered as identified at the species level when their sequence showed ≥99 % homology with a sequence deposited in GenBank. Sequences were also aligned with BioEdit v7.0.0 software (Hall 1999) for comparison.

Results

A total of 842 sand flies were collected: 574 by sticky papers and 268 by CDC miniature light traps (Table 1). Five species belong to the genus *Phlebotomus*: *Phlebotomus* (*Phlebotomus*) *papatasi* Scopoli, 1786, *P.* (*Paraphlebotomus*) *sergenti* Parrot, 1917, *P.* (*Pa.*) *caucasicus* Marzinovsky, 1917, *P.* (Pa). *mongolensis* Sinton, 1928 and *P.* (*Pa.*) *jacusieli* Theodor, 1947 and also 3 species belong to the genus *Sergentomyia*: *S. (Sergentomyia) dentata* Sinton, 1933, *S.* (*Se.*) *sintoni* Pringle, 1953 and *S.* (*Sintonius*) *clydei* Sinton, 1928 (Fig. 2).

The captured specimens by sticky papers show that *P. papatasi* is the most abundant species in this area (33%) followed by *P. sergenti* (22%) and *S. sintoni* (13%). The other species have relative abundances less than 10% (Table 1). The genus *Phlebotomus* (77.9%) is more abundant than the genus *Sergentomyia* (22.1%). The sex ratios show more males than females: 1.1 for the genus *Phlebotomus* and 1.9 for the genus *Sergentomyia*. The majority of these caught female specimens are unfed and gravid.

Of 268 specimens caught by CDC miniature light traps, the most prevalent sand fly species was *P. papatasi* (45%) followed by *P. sergenti* (26%). The sex ratios show more females than males (1.05) for the genus *Phlebotomus* and fewer females than males (0.75) for the genus *Sergentomyia*.

Most of the sand flies were collected from outdoor places (463: 55%) and in animal shelters (320: 38%). A few specimens have been caught indoors (59: 7%): *P. papatasi*, *P. sergenti*, Caucasicus group and *S. sintoni*. Of the specimens caught outdoors, 21 *P. caucasicus*/Caucasicus group, 19 *S. clydei*, 21 *S. sintoni* and 14 *S. dentata* were captured in rodent burrows.

The females consisted of 368 out of 842 (43.7%) caught specimens. Nine out of 151 (6%) female *P. papatasi* and three out of 60

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(5%) female Caucasicus group were infected by *L. major* and one out of 90 (1.1%) female *P. sergenti* tested for *Leishmania* DNA was also positive for *L. tropica* infection using the Standard PCR method. Each one of these specimens produced 430 bp band (Fig. 3). There was not any infected sample of female *Sergentomyia* sand flies in this study.

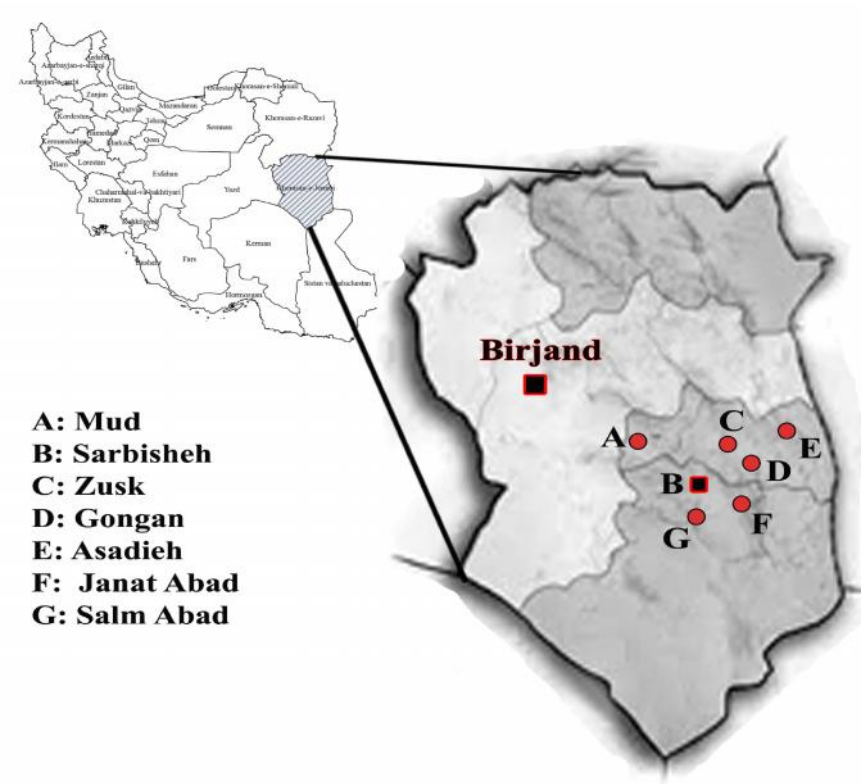

Fig. 1. Villages prospected in Sarbisheh County (Eastern Iran)

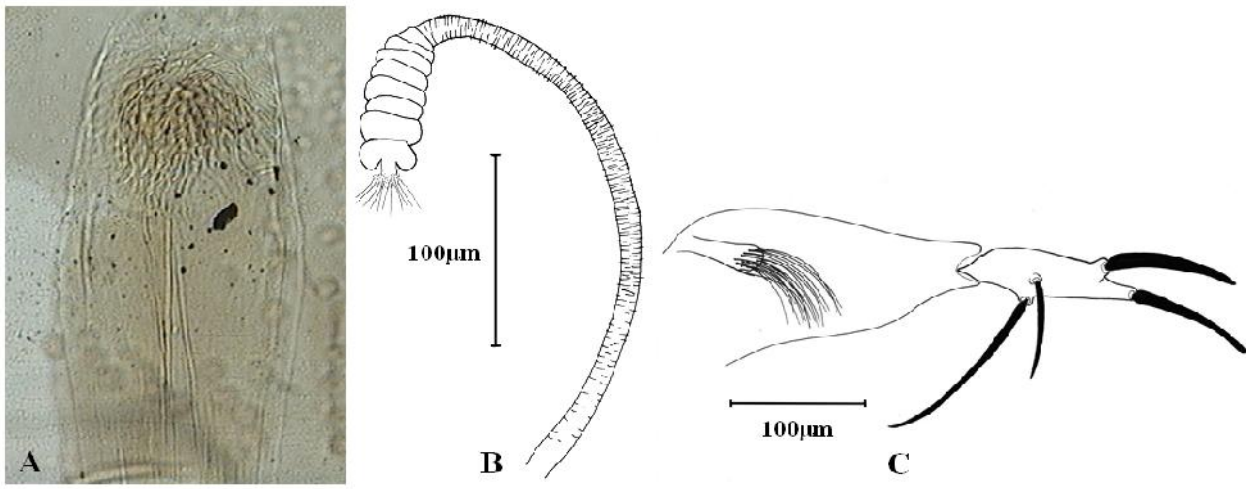

Fig. 2. *Phlebotomus jacusieli* caught in Sarbisheh. Female pharynx (A) and spermathecae (B), male coxite and style (internal view) (C)

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Published Online: August 31, 2013

Table 1. Caught samples of sand fly from different rural regions and various habitats of Sarbisheh County in Eastern Iran

County	Village	Habitat	Trap type	*Phlebotomus*				*Paraphlebotomus*					*Sergentomyia*						Total
				P. papatasi		*P. sergenti*		*P. caucasicus*	*P. mongolensis*	caucasicus group	*P. jacusieli*		*S. sintoni*		*S. dentata*		*S. clydei*		
				♂	♀	♂	♀	♂	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Sarbisheh	Gongan	A	S	6	5*	4	1	3	3	1	_	_	4	2	1	1	2	1	34
			C	2	4	2	3	_	_	1	_	_	1	1	_	2	_	_	16
		H	S	2	1	1	_	_	_	_	_	_	2	_	_	_	_	_	6
			C	1	1	1	_	_	_	_	_	_	_	_	_	_	_	_	3
		O	S	9	6*	4	3	2	3	3	2	_	6	2	2	1	2	1	46
			C	2	5	2	4	1	2	_	_	_	1	1	_	3	1	_	22
	Zusk	A	S	5	6	4	5	2	1	2	_	_	2	1	1	1	1	1	32
			C	2	_	3	3	_	_	1	_	_	1	_	_	1	1	1	13
		H	S	_	1	2	1	_	_	_	_	_	1	_	_	_	_	_	5
			C	1	_	_	_	_	_	_	_	_	_	_	_	_	_	_	1
		O	S	8	4	6	7	2	4	3	2	1	4	1	2	_	2	_	46
			C	4	4	4	3	2	2	1	_	_	_	1	1	1	_	_	23
	Mud	A	S	6	3	2	3	3	2	2	_	_	3	2	_	_	2	2	30
			C	3	2	3	2	_	_	_	_	_	1	1	_	1	_	1	14
		H	S	_	_	_	_	_	_	_	_	_	2	_	_	_	_	_	2
			C	2	1	_	_	_	_	_	_	_	_	_	_	_	_	_	3
		O	S	7	4	6	3	2	3	4	2	_	2	3	2	1	3	1	43
			C	5	5	2	3	1	1	1	_	_	1	1	1	_	_	_	21
	Janat abad	A	S	5	3	5	3	1	2	2	_	_	2	1	1	1	1	1	28
			C	4	7	2	2	_	_	1	_	_	_	_	1	_	1	_	18
		H	S	3	2	2	_	_	_	1	_	_	_	_	_	_	_	_	8
			C	1	_	_	2	_	_	_	_	_	_	_	_	_	_	_	3
		O	S	8	5*	6	5	1	3	3	_	_	3	2	3	1	2	_	42
			C	3	5	2	3	1	1	1	_	_	1	_	1	_	_	_	18
	Sarbisheh	A	S	4	6	3	1	2	2	2	_	_	3	1	1	_	1	2	28
			C	3	3	3	2	1	_	1	_	_	1	_	1	_	_	_	15
		H	S	3	3	1	_	_	_	_	_	_	1	_	_	_	_	_	8
			C	1	1	1	1	_	_	_	_	_	_	_	_	_	_	_	4
		O	S	9	4	5	6**	1	2	3	_	_	4	2	1	2	2	1	42
			C	2	5*	3	2	1	1	2	_	_	2	_	1	_	1	_	20
	Salm Abad	A	S	5	4	4	1	_	_	3	_	_	3	1	2	_	2	_	25
			C	3	4	2	1	1	1	1	_	_	1	1	_	_	_	_	15
		H	S	3	_	_	1	_	_	1	_	_	1	_	_	_	_	_	6
			C	1	_	1	_	_	_	_	_	_	_	_	_	_	_	_	2
		O	S	4	7*	5	6	2	2	6*	1	1	3	2	1	1	2	1	44
			C	3	5*	2	2	1	1	1	_	_	2	_	_	_	_	1	18
	Asadieh	A	S	7	9*	3	2	1	1	4*	_	_	2	1	2	1	_	1	34
			C	4	8	1	_	1	1	2	_	_	_	_	_	_	_	1	18
		H	S	2	2	_	_	_	_	1	_	_	1	_	_	_	_	_	6
			C	1	1	_	_	_	_	_	_	_	_	_	_	_	_	_	2
		O	S	11	9*	8	7	2	3	5*	2	1	4	1	2	1	2	1	59
			C	6	6*	_	2	1	1	1	_	_	1	_	_	_	1	_	19
Total				161	151	105	90	35	42	60	9	3	66	28	27	19	29	17	842

A: Animal Shelter, H: House, O: Outdoor, S: Sticky Paper, C: CDC miniature light trap

*: includes specimens infected by *L. major* **: includes specimen infected by *L. tropica*

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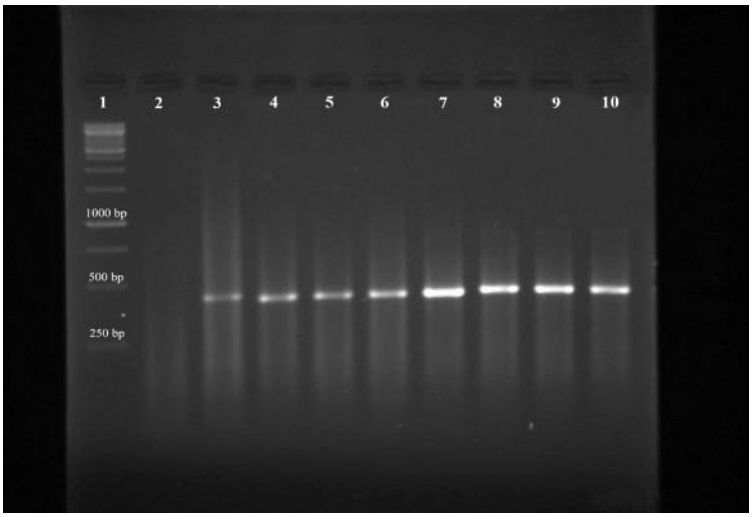

Fig. 3. Gel electrophoresis profile of the standard PCR based amplification products. The bands correspond to molecular weight marker (Lane1), control negative (Lane 2), reference strains of *L. major* (Lane 3) and *L. tropica* (Lane 4), *P. papatasi* (Bir-733: Lanes 5 and Bir-803: Lane 6), caucasicus group (Bir-211: Lane 7, Bir-341: Lane 8 and Bir-624: Lane 9) and *P. sergenti* (Bir-797: Lane 10)

Discussion

Little is known about sand fly fauna in Khorasan-e-Jonoubi Province. Several investigations have previously been carried out on the sand flies fauna in Khorasan district e.g. in Meshed, Lotf Abad, Esfarayen and Neishabur counties (Mesghali et al. 1967, Nadim et al. 1971, Javadian et al. 1976, Nadim and Tahvildar-Biruni 1977).

Nadim et al. (1971) reported a list of sand fly species caught in the mountains and plains of Khorasan district. They consisted of 12 species of *Phlebotomus* and 9 *Sergentomyia*. Out of the mentioned sand flies, some species were found only in the mountains and the caves (*P. major, S. pawlovskyi*). Others species were found both in mountains and in flat areas: *P. mongolensis*, *P. caucasicus*, *P. mofidii*, *P. ansarii, P. kandelakii* in the north Khorasan, *P. kazeruni*, *P. eleonorae*, *S. christophersi, S. tiberiadis* and *S. mervinae* near the central desert in the south of the central desert in the south of Khorasan district.

Nadim and Seyedi-Rashti (1972) have studied the sand fly fauna of the western part of the Khorasan district and recorded nine *Phlebotomus* and 10 *Sergentomyia* species: *P. papatasi*, *P. sergenti*, *P. caucasicus*, *P. alexandri*, *P. kazeruni*, *P. jacusieli*, *P. eleonorae*, *P. major*, *P. chinensis*, *S. sintoni*, *S. dentata*, *S. mervynae*, *S. grekovi*, *S. sumbarica*, *S. squamipleuris*, *S. pawlovskyi*, *S. clydei*, *S. tiberiadis* and *S. christophersi*. Moreover, *P. papatasi*, *P. sergenti*, females belonging to the Caucasicus group (*P. mongolensis* and *P. caucasicus*), *S. clydei* and *S. sintoni* have been found infected by promastigotes (Nadim and Seyedi-Rashti 1972).

Our findings are in accordance with the previous investigations carried out in this district (Nadim et al. 1971, Nadim and Seyedi-Rashti 1972). Since the 70's, no investigation has been carried out in this province and the Sarbisheh County has never been prospected.

Nine *P. papatasi* females and three females belonging to the Caucasicus group carried *L. major* DNA according to the BLAST process. The sequences obtained from the present study have been deposited in Genbank under accession numbers JN541326 to JN541337. These are identical or highly sim-

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Published Online: August 31, 2013

ilar to several sequences deposited in GenBank, including isolates from Iran and Friedlin references (Friedlin FR796423, Iran AY260965, AY283793, EF413075, GQ402544, GQ402543, GQ466354 and GQ466350).

The sequence of ITS-rDNA obtained from the female *P. sergenti* (accession number: JN541338) is similar (99% homology) to those *L. tropica* that previously have been deposited in GenBank from two regions of Iran: Shiraz (HM060588, HM060589, HM060590) (Oshaghi et al. 2010) and Kaleybar (EU604811, EU604813) (Parvizi and Ready 2008).

Investigation on the reservoirs of zoonotic cutaneous leishmaniasis (ZCL) was previously carried out by Seyedi-Rashti and Nadim (1967) in two regions of Khorasan district: Meshed (and its suburbs) and Lotf Abad. They mentioned *Rhombomys opimus* as the main reservoir of CL in the endemic focus of the rural type in Lotf Abad region. Later, rodent reservoirs of CL were studied in Meshed, Lotf abad, Sarakhs and Esferayen districts (Nadim and Seyedi-Rashti 1972, Javadian et al. 1976) and *Rhombomys opimus* was again reported as the main reservoir. The reservoirs of ZCL in Khorasan-e-Jonoubi Province due to insufficient studies in mentioned rural regions are not very clear. It seems that *R. opimus* is the main reservoir in this region as well as Khorasan district. In our study, 3 infected specimens by *L. major* have been captured in rodent burrows. Two of them were *P. papatasi* and the third one belonged to the Caucasicus group.

Sarbisheh County is located in the north of Sistan v Balouchestan Province, in border of Afghanistan. This area is separated from the central parts of Iran by deserts (Dasht-e-Kavir in north-east and Dasht-e-Loot in south-east).

Several investigations have been carried out in Sistan and Balouchestan both on vectors and reservoirs of cutaneous leishmaniases in some regions of this province (Kassiri et al. 2011a, 2011b, 2012).

Kassiri et al. (2011a) reported five species as proven (*P. papatasi*) or probable (*P. salehi*, *P. sergenti*, *P. alexandri* and *P. keshishiani*) vectors of cutaneous and visceral leishmaniasis in this province. They showed the role of *P. papatasi* and *P. salehi* in maintenance and transmission of *L. major* to humans and reported *Meriones hurrianae* and *Tatera indica* as the probable reservoirs of ZCL (Kassiri et al. 2011b, 2012).

To our knowledge, no study has been carried out on the leishmaniases in the province of Khorasan-e-Jonoubi. However, it seems that illegal immigration with low sanitary conditions occurs from Afghanistan and Pakistan, two countries where CL are endemic (Bhutto et al. 2009, Ruiz Postigo 2010) to Sarbisheh County. Consequently, there is a potential risk of leishmaniasis outbreak in this area, according to the presence of numerous *P. papatasi* and *P. sergenti*, in this area.

Several investigations show various ratios of females *P. papatasi* infected by *L. major* in following districts: 19.8% in Shiraz (Oshaghi et al. 2010), 15.6% in Badrood (Yaghoobi-Ershadi et al. 2005), 6.5% in Baft (Oshaghi et al. 2008), 11% in Damghan (Rassi et al. 2011), 12.5% in Shahrood (Abaei et al. 2007), 22.1% in Abardejh (Nekouie et al. 2006), 12.7% in Rafsanjan (Yaghoobi-Ershadi et al. 2010) and 2.1% in Chabahar (Kassiri et al. 2012). Our findings indicates 9 out of 151 (6%) *P. papatasi* carrying *L. major* DNA, constitute a relatively low level.

Concerning the females belonging to the Caucasicus group, 4.2 to 7.5% of the specimens were infected by *L. major* in several studies carried out in Borkhar, Ahar, Damghan and Shahrood districts (Yaghoobi-Ershadi et al. 1994, Rassi et al. 2004, Abaei et al. 2007, Rassi et al. 2011). In the present study, 3 out of 60 (5%) of females belonging to Caucasicus group have been found positive for *L. major* DNA, ranking these specimens in the mean of reported range in the mentioned areas of the country.

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Published Online: August 31, 2013

Cutaneous leishmaniasis due to *L. tropica* occurs in a vast distribution areas of the world. Its cycle varies between localities and generally does not require a sylvatic reservoir. Several surveys report the isolation of *L. tropica* from *P. sergenti*, its classical vector (Al-Zahrani et al. 1988, Oshaghi et al. 2010). However, in the middle-east, an atypical focus transmitted by *P.* (*Adlerius*) *arabicus* with reservoirs (hyraxes) has been recorded very close to classical focus (Svobodova et al. 2006). In eastern Africa, the subgenus *Larroussius* can also been implicated in the transmission of *L. tropica* (Lawyer et al. 1991). In Iran, *L. tropica* CL was first described in Tehran by Schlimmer several decades before the discovery of the parasite. Some of the most important foci of this disease are Tehran in center, Meshed in North-East, Shiraz and Kerman in south of Iran (Nadim and Seyedi-Rashti 1971).

Our pilot study reports a sand fly inventory showing that *P. papatasi* and *P. sergenti* are abundant species. The detection of *L. major* and *L. tropica* from the latter shows their local role in the transmission of the disease in emerging or previously unknown foci of cutaneous leishmaniases.

There is lack of knowledge in the prospected county about the local reservoirs and human prevalence and incidence. We suggest starting new studies at different periods of the year in order to explore all the counties of the province and to study the seasonal activity of Phlebotomine sand flies that our study cannot evaluate.

Acknowledgements

The authors are grateful to authorities of Pasteur Institute of Iran and Reims University, Parasitology departments for their valuable assistance and supporting of this investigation. We also thank Pr Nicole Léger for the confirmation of species identification. The authors declare that there is no conflict of interest.

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