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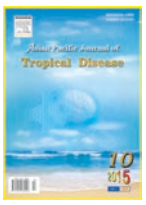

Original article doi: 10.1016/S2222-1808(15)60932-X

Faunal identification and frequency distribution of wild sand flies infected with *Leishmania tropica*

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ARTICLE INFO

Article history:
Received 7 Jul 2015
Received in revised form 21 Jul 2015
Accepted 28 Sep 2015
Available online 8 Oct 2015

Keywords:
Sand fly
Vector
Leishmania tropica
Zoonosis
Phlebotomus sergenti
Dry oriental sore
Iran

ABSTRACT

Objective: To determine the sand fly composition, its frequency distribution and natural infection with *Leishmania* parasites to incriminate the likely vectors of cutaneous leishmaniosis in Jiroft, Southeast Iran.
Methods: Sand flies were caught with sticky paper traps during a 6-month period in 2013. They were morphologically identified to species level using valid taxonomic keys. They were then subjected to nested PCR method and the results were analyzed.
Results: A total of 3 751 sand flies were identified to belong to 21 species in two genera (8 spp. in the *Phlebotomus* genus, and 13 spp. in the *Sergentomyia* genus) most of which were males (65.60%) and exophilic (63.80%). The two most frequent species were *Phlebotomus papatasi* (39.40%) and *Phlebotomus sergenti* (17.20%). The latter was confirmed by PCR to be naturally infected with *Leishmania tropica* (3.33%).
Conclusions: It was thus concluded that wild-caught *Phlebotomus sergenti* naturally infected with *Leishmania tropica* was mainly incriminated by molecular method to be the principal vector of cutaneous leishmaniosis in this endemic focus.

1. Introduction

The flagellate parasites *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatidae) are the causative agents of leishmaniasis (or leishmaniosis), a complex of protozoan infections naturally transmitted to mammals, including human beings, by the bites of female phlebotomine (Diptera: Psychodidae, subfamily Phlebotominae) sand flies[1]. Primary skin infections [cutaneous leishmaniasis (CL)] often resolve without any treatments, but they can produce secondary lesions in the mucosa [mucocutaneous leishmaniasis (ML)] and the viscera (visceral leishmaniasis, which is fatal if left untreated)[2,3]. This condition could be further exacerbated if simultaneous co-infection with other vector-borne pathogens occurred[4].

Leishmaniasis is still one of the most neglected tropical diseases being endemic in 98 countries with an estimated prevalence of 12 million people, annual incidence of about 2 million new cases and 50000 deaths per year due to visceral leishmaniasis[2]. CL accounts for 75% of the total cases, 90% of which occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Bolivia, Brazil, Colombia, Nicaragua and Peru[2].

Though not fatal, CL has a significant social impact on patients. It has been endemic in the Old World since ancient times. It occurs in two epidemiologic forms: anthroponotic CL (ACL) and zoonotic CL. The latter is caused by *Leishmania major* Yakimoff & Schokhor (*L. major*) mainly in rural areas where gerbils are the main rodent reservoir hosts and wild sand flies like *Phlebotomus papatasi* (Scopoli, 1786) (*P. papatasi*), as proven vectors[5-8]. In contrast, ACL due to *Leishmania tropica* (Wright, 1903) (*L. tropica*) occurs in densely populated urban settings and humans appear to be the main reservoir hosts. Zoonotic CL is well distributed in suburban or remote areas of at least 17 out of 31 provinces, whereas ACL appears to be restricted to urban foci of only 8 provinces in Iran[9].

The blood sucking sand fly *Phlebotomus sergenti s.l.* (Parrot, 1917) (*P. sergenti*) is widely distributed in Iran where it is the main definitive vector transmitting *L. tropica*, the causative agent of ACL in the Old

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World[1], albeit in some other areas specific sand flies (*Phlebotomus arabicus* and *Phlebotomus guggisbergi*) may play a role in its transmission. The geographical distribution of medically important *P. sergenti*, which is found in 26 out of 31 provinces of Iran, is much wider than that of *L. tropica*. The latter is generally unresponsive to the main line of treatment with glucantime drug in human host[10], runs the potential risk of becoming viscerotropic[11,12], and significantly increases in CL incidence leading to the expansion of the disease to new foci particularly after natural disasters[13-15].

Following a recent study on the prevalence and parasite species identification of CL among people in the city of Mohammadabad, Jiroft County, Kerman Province, Southeast Iran[16], the present study was performed in endemic areas of Jiroft on faunal identification and frequency distribution of sand flies naturally infected with *L. tropica* using PCR method to detect kinetoplast DNA (kDNA) of parasites. To the best of authors' knowledge, this is the first report on detection of *L. tropica* from sand flies in this endemic area. It is noteworthy how few studies of *L. tropica* from sand flies have been published so far from Iran and its adjacent countries.

2. Materials and methods

2.1. Study areas

The present research was conducted in the county town of Jiroft (28°40' N, 57°45' E), Kerman Province (Figure 1). Jiroft lies on the southern part of Kerman Province and covers approximately 13 799 km^2 of land. It consists of two mountainous and plateau parts. Three different climates prevail with cold Sardouieh, mild Barez and Dalfard mountains in the north and hot humid desert areas in the central and southern parts of the county. The altitude varies from 680 m in the city of Jiroft to 3886 m on Sardouieh heights. Its mean annual temperature is 27 ℃. This study was performed from May to November 2013 during the peak seasonal density period of sand fly around nine representative villages (Mohammadabad, Asbi, Derdsk, Saghder, Dashtkooch, Thouhan, Jangalabad, Miandeh and Mianchill) in the Jiroft County. These localities were the best chosen on the basis of previous reports of CL cases.

2.2. Sand fly collection and species identification

Sand flies were caught using sticky paper traps indoors and outdoors from nine named villages of Jiroft County. For each sampling, sand flies were collected once every fortnight from indoor habitats (bedrooms, bathrooms, storage rooms, *etc*.) and outdoor habitats (rodent burrows, below rocks, stone and wall crevices, *etc*.). In each round of sampling 60 sticky paper traps (30 indoors, 30 outdoors) were mounted after sunset and removed every morning after. Each trap laid on one night was taken a "trap-night" and there were 120 "trap-nights"/month. Male sand flies were detached using acetone and fine entomologic needles. They were then identified to species level with the aid of valid taxonomic keys. They were kept in 70% ethanol for mounting and species identification, while female sand flies were processed for kDNA extraction.

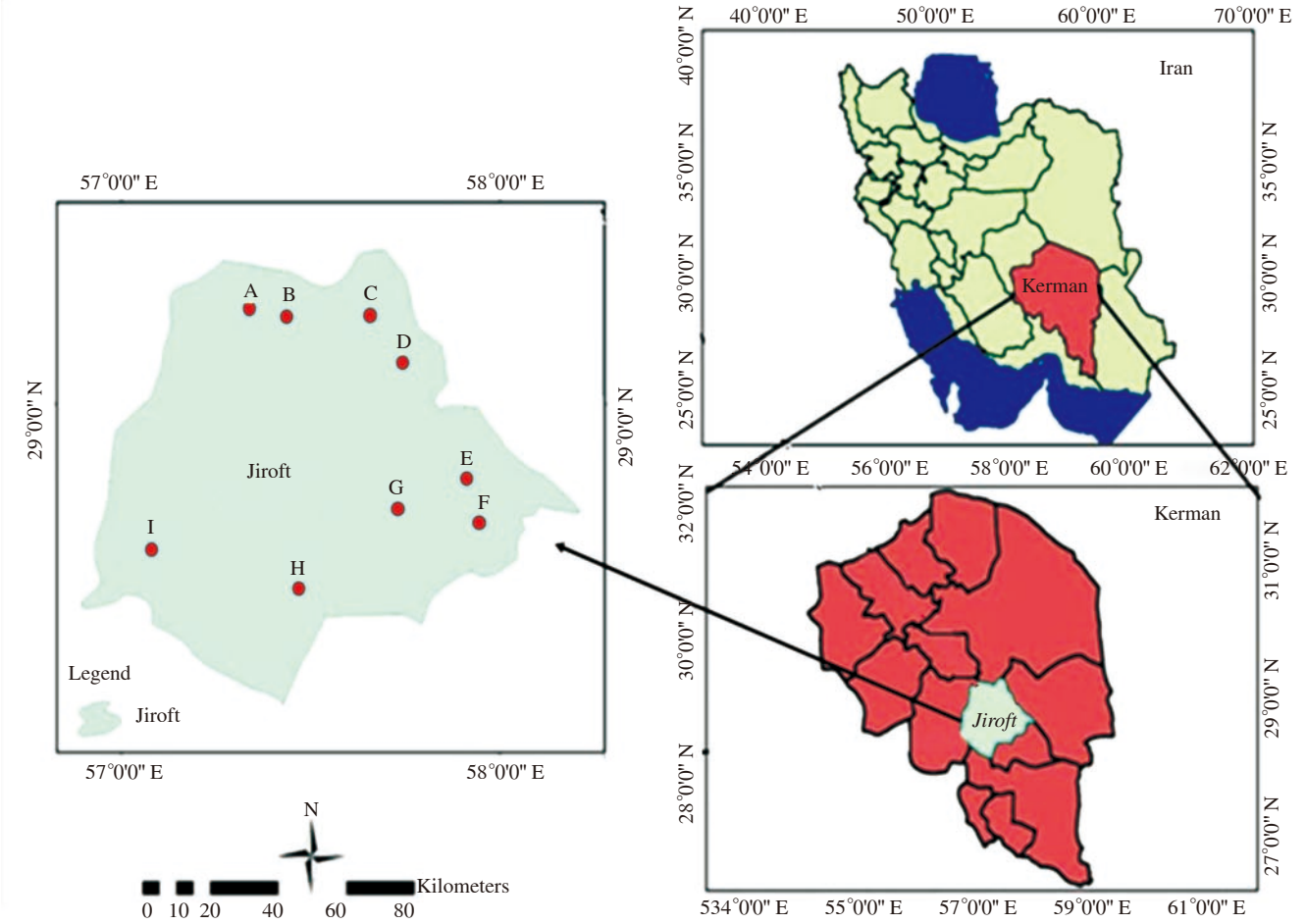

Figure 1. Map of the study areas showing locations of nine villages in Jiroft County, Kerman Province, Southeast Iran.
A: Mohammadabad; B: Asbi; C: Derdsk; D: Saghder; E: Dashtkooch; F: Thouhan; G: Jangalabad; H: Miandeh; I: Mianchill.

Using two needles, the heads and last two abdominal segments from female sand flies were soaked in a drop of Puri's medium on a microscope slide to identify each sand fly to species level based on the taxonomic keys[6]. The remaining body parts of previously blood-fed and digested (void) uniparous females of the common phlebotomine sand fly species [*P. papatasi*, *P. sergenti*, and *Phlebotomus alexandri* (*P. alexandri*)] were used for DNA extraction and later PCR processing. Parous female sand flies were distinguished from nulliparous individuals by the presence or absence of yellow granules in the accessory glands. In contrast, nulliparous females had a well-developed volume of fat bodies which was reflected in the specific gravity. Thus, young female sand flies floated in saline solution in which older ones sanked.

2.3. DNA extraction

DNA extraction from sand flies was conducted with the nested PCR procedure as described before[6]. Initially, the thorax and anterior abdominal segments of dissected sand flies were ground and homogenized in 96% ethanol within 1.5 mL Eppendorf® microtubes with fine glass pestle. The homogenate was then added to 200 μL buffer [50 mmol/L Tris-HCl (pH 7.6), 1 mmol/L ethylene diamine tetraacetic acid, 1% Tween 20] containing 5 μL proteinase K solution (20 μg/mL) in a microtube. This was then incubated for 24 h at 37 ℃ before 50 μL phenol: chloroform: isoamyl alcohol (25:24:1, by volume) were added. This was then well shaken, settled for 5 min and centrifuged at 15000 r/min for 10 min at 4 ℃. Following centrifugation, the DNA in the supernatant solution was precipitated with 200 μL pure ethanol. It was then desiccated at 37 ℃ with open lid, re-suspended in 100 μL ddH_2O, desiccated and stored at 4 ℃ before being used in molecular assay.

2.4. Detection and identification of Leishmania species

The variable segment on minicircles of kDNA from any *Leishmania* parasites present in sand flies was amplified with two-round, nested PCR. The primers were designed within the conserved area of the minicircle kDNA which included conserved sequence blocks (CSB) of CSB1XR (CGA GTA GCA GAA ACT CCC GTT CA) and CSB2XF (ATT TTT CGC GAT TTT CGC AGA ACG) for the first round and LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATA G) for the second round.

Nested PCR protocol was implemented to amplify the variable region of the minicircle kDNA of any *Leishmania* in the sand fly midgut. It comprised a two-round nested PCR in which the two rounds were performed in separate test tubes and 2 μL of the first round product diluted with ddH_2O (4:1) was taken as template for the second round (nested) process.

The first round amplification reaction mixture had 250 μmol/L of deoxynucleoside triphosphate, 1.5 mmol/L $MgCl_2$, 1.0 IU of *Taq* polymerase, 50 mmol/L Tris-HCl (pH 7.6), 1% Tween 20, and 40 ng each of primers CSB1XR and CSB2XF, in a final volume of 25 μL. This reaction mixture was incubated in a CG1-96 thermocycler (Hamburg, Germany) set to run 30 cycles, each one consisting of incubation at 94 ℃ for 5 min, 55 ℃ for 1 min, 72 ℃ for 1 min, and finally 70 ℃ for 7 min and kept at 4 ℃.

The second-round nested amplification was conducted in a separate test tube containing a final volume of 25 μL reaction mixture with 1.0 IU of *Taq* polymerase buffer, $MgCl_2$, deoxynucleoside triphosphate added to 1 μmol/L primer LiR, 1 μmol/L primer 13Z, and 2 μL of the first amplification reaction mixture diluted 4:1 in ddH_2O. The PCR amplification product was then run for 33 cycles.

Negative controls (DNA extracts from male sand flies and aliquots of ddH_2O) were used in each PCR protocol to detect probable contamination which could produce false positive results, all were negative.

2.5. Standard strains of different Leishmania species

For comparison, standard reference strains of *L. major* (MHOM/IR/54/LV39), *L. tropica* (MHOM/IR/89/ARD2) and *Leishmania infantum* (*L. infantum*) (MCAN/IR/96/LON49) were used.

2.6. Agarose gel electrophoresis

A 5 μL sample of each second-round PCR end product was applied to electrophoresis in 1.5% agarose gel. The DNA bands were stained with 1% ethidium bromide, visualized on an UV transilluminator, and compared with molecular-weights markers and the relevant second-round products for the *L. major*, *L. tropica*, and *L. infantum* standards.

3. Results

A total of 3751 individual sand flies were captured using sticky paper traps. Most (2460, 65.60%) of them were male, while a smaller percentage (1291, 34.40%) were female (Table 1). Male sand flies were thus caught more than females in all sampled species except *Phlebotomus bergeroti* (*P. bergeroti*), *Sergentomyia baghdadis* (*S. baghdadis*), *Sergentomyia squamipleuris* (*S. squamipleuris*). The captured male/female ratio approximated to 2:1. Most sand flies were captured outdoors (63.80%). All except three species [two *Phlebotomus halepensis* (*P. halepensis*) and *P. major* group and one *Sergentomyia sogdania* species] were more frequently scattered outdoors than indoors.

Table 1

Frequencies of different sand fly species according to their sex and indoor/outdoor captivity in Jiroft County, Kerman Province, Southeast Iran. *n* (%).

Species	Sex		Trap		Total
	Male	Female	Outdoor	Indoor	
P. papatasi	1004 (67.80)	475 (32.20)	894 (60.40)	585 (39.60)	1479 (39.40)
P. sergenti	488 (75.80)	156 (24.20)	386 (59.90)	258 (40.10)	644 (17.20)
P. salehi	1 (100.00)	0 (0.00)	1 (100.00)	0 (0.00)	1 (0.03)
P. bergeroti	0 (0.00)	14 (100.00)	14 (100.00)	0 (0.00)	14 (0.37)
P. alexandri	117 (68.90)	53 (31.10)	78 (45.90)	92 (54.10)	170 (4.50)
P. halepensis	3 (100.00)	0 (0.00)	3 (100.00)	0 (0.00)	3 (0.08)
P. major[*]	7 (100.00)	0 (0.00)	0 (0.00)	7 (100.00)	7 (0.19)
P. ansarii	1 (100.00)	0 (0.00)	0 (0.00)	1 (100.00)	1 (0.03)
Sergentomyia sintoni	65 (72.30)	25 (27.70)	55 (61.10)	35 (38.90)	90 (2.40)
S. baghdadis	141 (33.80)	276 (66.20)	288 (69.00)	129 (31.00)	417 (11.10)
Sergentomyia grekovi	30 (79.00)	8 (21.00)	21 (55.20)	17 (44.80)	38 (1.01)
Sergentomyia tiberiadis	78 (69.00)	35 (31.00)	89 (78.70)	24 (21.20)	113 (3.01)
S. dentata	191 (75.40)	62 (24.60)	167 (66.00)	86 (34.00)	253 (6.75)
Sergentomyia africana	2 (66.70)	1 (33.30)	3 (100.00)	0 (0.00)	3 (0.08)
Sergentomyia antenata	91 (84.20)	17 (15.80)	72 (66.66)	36 (33.30)	108 (2.88)
Sergentomyia palestinensis	44 (83.00)	9 (17.00)	31 (58.50)	22 (41.50)	53 (1.40)
S. squamipleuris	7 (36.90)	12 (63.10)	16 (84.20)	3 (15.80)	19 (0.50)
S. clydei	175 (55.80)	139 (44.20)	264 (84.00)	50 (16.00)	314 (8.37)
Sergentomyia sogdiana	13 (59.10)	9 (40.90)	9 (41.00)	13 (59.00)	22 (0.59)
Sergentomyia theodori	1 (100.00)	0 (0.00)	1 (100.00)	0 (0.00)	1 (0.03)
Sergentomyia sumbarica	1 (100.00)	0 (0.00)	1 (100.00)	0 (0.00)	1 (0.03)
Total	2460 (65.60)	1291 (34.50)	2393 (63.80)	1358 (36.20)	3751 (100.00)

[*]: *P. major* group, species unidentified. *S. clydei: Sergentomyia clydei; S. dentata: Sergentomyia dentata; P. salehi: Phlebotomus salehi; P. ansarii: Phlebotomus ansarii.*

Furthermore, in the present study, two distinct genera (*Phlebotomus*, *Sergentomyia*) or 21 different phlebotomine sand fly species (8 *Phlebotomus* and 13 different *Sergentomyia* species) were morphologically identified to species level following capture by sticky paper traps. Of these, the genus *Phlebotomus* represented by 2319 (61.80%) individuals were thus considered to be more common. The three most numerously captured *Phlebotomus* species were *P. papatasi* (39.40%), followed by *P. sergenti* (17.20%) and *P. alexandri* (4.50%), while the three most frequently found *Sergentomyia* species were *S. baghdadis* (11.10%) followed by *S. clydei* (8.37%) and *S. dentata* (6.75%).

From Table 1, it was observed that among *Phlebotomus* sand flies, no male *P. bergeroti* and no female *P. salehi*, *P. halepensis*, *P. ansarii*, or *P. major* group was caught. In addition, no *P. ansarii* and *P. major* group was captured outdoors. On the other hand, no *P. salehi*, *P. bergeroti*, or *P. halepensis* was found to be endophilic (indoors).

The frequencies of different sand fly species with respect to their geographical positions were indicated in Table 2. Most (1270, 33.86%) sand flies were caught in Dashtkooch Village, while the lowest number of sand flies (54, 1.44%) were captured from Jangalabad Village. Most of those captured in Dashtkooch belonged to *P. papatasi* sand fly species, whereas no *P. sergenti* were found in the same area. This sand fly species was mainly caught in Saghder, Asbi, and Mohammadabad.

The results of molecular typing revealed that only two (1.67%) *P. sergenti* out of 120 unfed uniparous female phlebotomine sand flies tested for *Leishmania* parasites by being subjected to nested PCR method against kDNA were positive for *L. tropica* parasites (Figure 2). The only parasite identified was *L. tropica* with a main band of 750 bp distinct from those of *L. major* (560 bp) and *L. infantum* (680 bp). The kDNA bands for *L. tropica* in *P. sergenti* exhibited a thinner width or weaker staining than those for *L. infantum* (Lane 2). Both *P. papatasi* and *P. alexandri* were shown to be negative to *Leishmania* parasites in this region (Table 3).

Table 2

Frequencies of different sand fly species according to their geographical locations and sex in Jiroft County, Kerman Province, Southeast Iran.

Species		Sex	Thouhan		Asbi		Jangalabad		Derdsk		Miandeh		Dashtkooch		Saghder		Mianchill		Mohammadabad		Total
			O	I	O	I	O	I	O	I	O	I	O	I	O	I	O	I	O	I	
Phlebotomus	*P. papatasi*	Male	89	40	9	6	8	14	0	0	45	20	386	294	22	8	35	20	8	3	1004
		Female	98	20	5	0	6	1	0	0	15	3	154	147	2	3	10	5	6	0	475
	P. salehi	Male	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	P. bergeroti	Male	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Female	8	0	0	0	0	0	0	0	3	0	3	0	0	0	0	0	0	0	14
Paraphlebotomus	*P. alexandri*	Male	3	18	1	13	1	0	0	1	4	11	14	11	11	12	8	2	4	1	117
		Female	0	3	3	2	3	0	0	2	7	0	8	4	5	1	7	1	0	0	53
	P. sergenti	Male	0	0	44	30	8	0	3	5	0	0	0	0	178	135	0	0	59	26	488
		Female	0	0	38	25	1	0	1	4	0	0	0	0	33	16	0	0	21	17	156
Synphlebotomus	*P. ansarii*	Male	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Larroussius	*P. major*[*]	Male	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	7
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adlerius	*P. halepensis*	Male	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Grassomyia	*S. squamipleuris*	Male	0	0	0	0	0	0	0	0	0	0	1	0	3	1	0	0	2	0	7
		Female	0	0	0	0	0	0	0	0	1	0	8	1	0	1	1	0	0	0	12
Sintonius	*S. clydei*	Male	11	17	0	1	0	0	2	2	82	1	30	13	8	0	8	0	0	0	175
		Female	13	12	0	0	0	0	0	0	66	1	32	3	2	0	10	0	0	0	139
	Sergentomyia tiberiadis	Male	16	0	0	0	0	0	0	0	32	0	15	10	0	0	0	0	0	0	78
		Female	5	0	0	0	0	0	0	0	18	0	3	3	0	0	0	0	0	0	35
Sergentomyia	*Sergentomyia antenata*	Male	1	0	28	6	0	0	0	0	1	0	1	1	11	7	0	0	25	10	91
		Female	0	0	4	5	0	0	0	1	0	0	0	2	0	2	0	0	1	2	17
	S. dentata	Male	4	0	50	10	3	2	6	6	7	7	5	9	13	14	0	0	41	14	191
		Female	2	0	7	2	0	2	0	5	0	0	1	1	14	8	0	0	14	6	62
	Sergentomyia theodori	Male	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sergentomyia sintoni	Male	4	1	4	6	1	1	2	2	0	0	4	2	6	7	0	0	17	5	65
		Female	2	2	3	5	1	1	0	0	0	0	0	2	4	1	0	0	4	0	25
Parrotomyia	*Sergentomyia africana*	Male	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	2
		Female	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
	S. baghdadis	Male	2	1	10	8	0	0	0	3	1	0	11	14	40	10	10	9	14	8	141
		Female	3	2	39	17	0	0	5	4	2	0	14	18	29	9	8	0	100	26	276
	Sergentomyia sumbarica	Male	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
		Female	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sergentomyia palestinensis	Male	10	15	2	1	0	0	1	0	5	0	8	1	1	0	0	0	0	0	44
		Female	2	0	0	0	0	0	0	0	0	0	2	0	0	4	0	0	0	1	9
	Sergentomyia sogdiana	Male	0	5	4	0	0	0	0	0	2	0	4	2	0	0	0	0	0	0	13
		Female	0	2	3	0	0	0	0	0	1	0	2	4	0	0	0	0	0	0	9
	Sergentomyia grekovi	Male	7	0	0	0	0	0	0	0	7	5	3	8	0	0	0	0	0	0	30
		Female	0	0	0	0	0	0	0	0	0	0	4	4	0	0	0	0	0	0	8

O: Outdoors; I: Indoors.

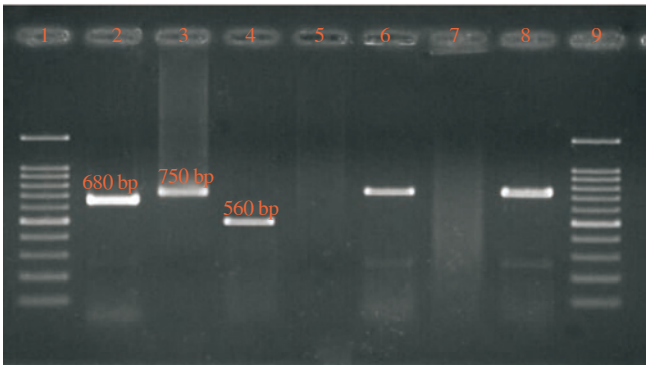

Figure 2. An illustration of the results of PCR products of kDNA from *Leishmania* parasites detected in sand flies.
Lanes 1 and 9: Molecular weight marker (100 bp); Lane 2: *L. infantum*; Lane 3: Reference strains of *L. tropica*; Lane 4: *L. major*; Lane 5: Negative controls (double-distilled water); Lanes 6, 7 and 8: Samples investigated came from wild-caught *P. sergenti*.

Table 3
Frequency distribution of three different sand fly species subjected to nested PCR assay and their percentage positivity for the presence of *L. tropica* parasites' kDNA. *n* (%).

Species	Total females	Investigated	Found infected
P. sergenti	156	60 (38.46)	2 (3.33)
P. papatasi	475	50 (10.52)	0 (0.00)
P. alexandri	53	10 (18.86)	0 (0.00)
Total	684	120 (17.54)	2 (1.67)

4. Discussion

Leishmaniasis is the second most important vector-borne disease after malaria in Iran[17], although other infectious and non-infectious diseases also prevail in this region[18-21]. The main aims of the present study were to identify the sand fly vectors and causative agents of recent ACL cases in Jiroft County, Kerman Province of Southeast Iran, which is an endemic focus of *L. tropica* parasites in native people. It was found that wild-caught *P. sergenti* naturally infected with *L. tropica* was partly incriminated by molecular method to be the probable principal vector of human CL (ACL) in this new focus of Iran. Some of the most important ACL foci in the world include the cities of Kabul (Afghanistan), Mosul (Iraq), Ashkhabad (Turkmenistan), Aleppo (Syria), Sanliurfa (Turkey), Shiraz, Yazd and Bam in Iran[2,9,13,22-24].

The importance of the present study is partly reflected the fact that the county town of Jiroft lies next to the county town of Bam, where the famous earthquake of 2003 resulted in approximately 30 000 deaths[15]. This is the first evidence on isolation, detection and identification of *L. tropica* from *P. sergenti* sand flies in the endemic area of Jiroft, Southeast Iran. It is noteworthy how few reports of *L. tropica* from vector sand flies have been published so far. There have been a few sporadic publications on the detection and characterization of *L. tropica* from *P. sergenti* sand flies in the last decade. These included studies in Southwest Asian countries: Iran[9,13], Afghanistan[25], and Turkey[26]. and at least three African countries: Morocco[27,28], Tunisia[29] and Ethiopia[30]. Apart from these few studies, the sand fly vectors of *L. tropica* parasites remain ambiguous in all other ACL foci. In most cases, *P. sergenti* has been suggested to be the principal vector based on its high captured number and ecological profile.

In the present study, vector incrimination criteria for *P. sergenti* were partially achieved, since 1) wild blood-fed uniparous female sand flies were used to detect kDNA of *L. tropica* parasites; 2) parasite isolate from wild sand fly was identical with reference strain from patients; 3) the sand fly transmitted the parasite to people by nocturnal bite; 4) the sand fly fed on man. The latter two criteria emanated from personal experiences due to the residence of one of the authors in the endemic area as well as support from previously published literature[31].

The transmission cycle of ACL varies between disparate territories. It usually involves a spatio-temporal overlap of ACL disease cases with *L. tropica* infected sand flies transmitting the protozoan parasites either between human hosts via sand flies in epidemiologically susceptible congested settings (anthroponotic cycle) or from a putative wild reservoir host (such as canines, black rats or rock hyraxes, in a zoonotic cycle) via vectors to sparsely-spaced humans in remote areas[32]. In many cases, as in the present study, it is thought that human activity can increase the probability of disease emergence by a variety of interacting environmental routes of transmission, since most people move in summer from warm areas to colder mountainous parts to avoid hot climate.

There is thus an urgency to investigate and monitor each situation carefully. For instance, the sand flies *P. halepensis* were reported to be permissive to both *L. tropica* and *L. major* under experimental conditions[33]. It would thus be informative if any females of *P. halepensis* were to be found positive for *L. tropica* kDNA under field conditions. No female sand flies of this species were, however, caught in the present study.

The sand fly vector, *P. sergenti*, was also postulated to consist of a complex of sibling species, but this was later questioned using different molecular means (including cytochrome b sequencing) to repudiate the concept of siblings in this species[34].

It is thus concluded that in an endemic area of Southeast Iran, where clinical cases of CL have indicated the presence of *L. tropica* in patients, sand flies of *P. sergenti* were also shown by nested PCR assay to be positive with this protozoan parasites. *P. sergenti* is a principal vector of *L. tropica* in Jiroft, Iran.

Conflict of interest statement

We declare that we have no conflict of interests.

Acknowledgments

The present paper was extracted from the results of an approved MSc student thesis (No: 92-6706 Dated 29 Sep 2013) conducted by the second author, Mr. M. Ahmadyousefi-Sarhadi. Thanks are due to the Vice-Chancellor for Research and Technology at Shiraz University of Medical Sciences, for permitting the use of facilities at the university.

References

[1] Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *Lancet Infect Dis* 2007; **7**: 581-96.

[2] World Health Organization. Control of leishmaniasis. Geneva: World Health Organization; 2007. [Online] Available from: http://www.who.int/neglected_diseases/mediacentre/WHA_60.13_Eng.pdf [Accessed on 18th August, 2015]

[3] Shirian S, Oryan A, Hatam GR, Daneshbod Y. Three *Leishmania*/*L.* species-*L. infantum*, *L.major*, *L. tropica*-as causative agents of mucosal

leishmaniasis in Iran. *Pathog Glob Health* 2013; **107**(5): 267-72.

[4] Moemenbellah-Fard MD, Shahriari B, Azizi K, Fakoorziba MR, Mohammadi J, Amin M. Faunal distribution of fleas and their blood-feeding preferences using enzyme-linked immunosorbent assays from farm animals and human shelters in a new rural region of southern Iran. *J Parasit Dis* 2014; doi: 10.1007/s12639-014-0471-1.

[5] Moemenbellah-Fard MD, Kalantari M, Rassi Y, Javadian E. The PCR-based detection of *Leishmania major* infections in *Meriones libycus* (Rodentia: Muridae) from Southern Iran. *Ann Trop Med Parasitol* 2003; **97**(8): 811-6.

[6] Azizi K, Abedi F, Moemenbellah-Fard MD. Identification and frequency distribution of *Leishmania* (*L.*) *major* infections in sand flies from a new endemic ZCL focus in southeast Iran. *Parasitol Res* 2012; **111**: 1821-6.

[7] Azizi K, Moemenbellah-Fard MD, Kalantari M, Fakoorziba MR. Molecular detection of *Leishmania major* kDNA from wild rodents in a new focus of zoonotic cutaneous leishmaniasis in an oriental region of Iran. *Vector Borne Zoonotic Dis* 2012; **12**(10): 844-50.

[8] Azizi K, Fakoorziba MR, Jalali M, Moemenbellah-Fard MD. First molecular detection of *Leishmania major* within naturally infected *Phlebotomus salehi* from a zoonotic cutaneous leishmaniasis focus in Southern Iran. *Trop Biomed* 2012; **29**: 1-8.

[9] Aghaei AA, Rassi Y, Sharifi I, Vatandoost H, Mollaie H, Oshaghi MA, et al. First report on natural *Leishmania* infection of *Phlebotomus sergenti* due to *Leishmania tropica* by high resolution melting curve method in Southeastern Iran. *Asian Pac J Trop Med* 2014; **7**(2): 93-6.

[10] Hadighi R, Mohebali M, Boucher P, Hajjaran H, Khamesipour A, Ouellette M. Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. *PLoS Med* 2006; **3**(5): e162.

[11] Jacobson RL. *Leishmania tropica* (Kinetoplastida: Trypanosomatidae)-a perplexing parasite. *Folia Parasitol (Praha)* 2003; **50**: 241-50.

[12] Alborzi A, Rasouli M, Shamsizadeh A. *Leishmania tropica*-isolated patient with visceral leishmaniasis in Southern Iran. *Am J Trop Med Hyg* 2006; **74**: 306-7.

[13] Oshaghi MA, Rasolian M, Shirzadi MR, Mohtarami F, Doosti S. First report on isolation of *Leishmania tropica* from sandflies of a classical urban cutaneous leishmaniasis focus in Southern Iran. *Exp Parasitol* 2010; **126**: 445-50.

[14] Fakoorziba MR, Baseri A, Eghbal F, Rezaee S, Azizi K, Moemenbellah-Fard MD. Post-earthquake outbreak of cutaneous leishmaniasis in a rural region of Southern Iran. *Ann Trop Med Parasitol* 2011; **105**(3): 217-24.

[15] Aflatoonian MR, Sharifi I, Hakimi Parizi M, Fekri AR, Aflatoonian B, Sharifi M, et al. A prospective cohort study of cutaneous leishmaniasis risk and opium addiction in South Eastern Iran. *PLoS One* 2014; **9**(2): e89043.

[16] Poursmaelian S, Mirzaei M, Sharifi I, Zarean M. The prevalence of cutaneous leishmaniasis in the city and suburb of Mohammadabad, Jiroft District and identification of parasite species by nested-PCR, 2008. *J Kerman Univ Med Sci* 2011; **18**(3): 218-27.

[17] Moemenbellah-Fard MD, Saleh V, Banafshi O, Dabaghmanesh T. Malaria elimination trend from a hypo-endemic unstable active focus in southern Iran: predisposing climatic factors. *Pathog Glob Health* 2012; **106**(6): 358-65.

[18] Fakoorziba MR, Neghab M, Alipour H, Moemenbellah-Fard MD. Tick borne Crimean-Congo haemorrhagic fever in Fars Province, Southern Iran: epidemiologic characteristics and vector surveillance. *Pak J Biol Sci* 2006; **9**(14): 2681-4.

[19] Fakoorziba MR, Golmohammadi P, Moradzadeh R, Moemenbellah-Fard MD, Azizi K, Davari B, et al. Reverse transcription PCR-based detection of Crimean-Congo hemorrhagic fever virus isolated from ticks of domestic ruminants in Kurdistan Province of Iran. *Vector Borne Zoonotic Dis* 2012; **12**(9): 794-9.

[20] Fakoorziba MR, Naddaf-Sani AA, Moemenbellah-Fard MD, Azizi K, Ahmadnia S, Chinikar S. First phylogenetic analysis of a Crimean-Congo hemorrhagic fever virus genome in naturally infected *Rhipicephalus appendiculatus* ticks (Acari: Ixodidae). *Arch Virol* 2015; **160**(5): 1197-209.

[21] Hassanzadeh J, Mohammadbeigi A, Eshrati B, Moemenbellah-Fard MD. Estimation of the regional burden of non-communicable diseases due to obesity and overweight in Markazi Province, Iran, 2006-2007. *J Cardiovasc Dis Res* 2012; **3**: 26-31.

[22] Reyburn H, Rowland M, Mohsen M, Khan B, Davies C. The prolonged epidemic of anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: 'bringing down the neighbourhood'. *Trans R Soc Trop Med Hyg* 2003; **97**: 170-6.

[23] Reithinger R, Mohsen M, Leslie T. Risk factors for anthroponotic cutaneous leishmaniasis at the household level in Kabul, Afghanistan. *PLoS Negl Trop Dis* 2010; **4**(3): e639.

[24] Jacobson RL. Leishmaniasis in an era of conflict in the Middle East. *Vector Borne Zoonotic Dis* 2011; **11**(3): 247-58.

[25] Faulde M, Schrader J, Heyl G, Amirih M. Differences in transmission seasons as an epidemiological tool for characterization of anthroponotic and zoonotic cutaneous leishmanisis in Northern Afghanistan. *Acta Trop* 2008; **105**: 131-8.

[26] Pazarbasi A, Alptekin D, Luleyap HU, Kasap M, Kasap H. Use of enzyme-linked immunosorbent assay for detection of natural *Leishmania* infections in phlebotomine sand flies from Southeastern Turkey. *J Med Entomol* 2006; **43**: 248-51.

[27] Ajaoud M, Es-Sette N, Hamdi S, El-Idrissi AL, Riyad M, Lemrani M. Detection and molecular typing of *Leishmania tropica* from *Phlebotomus sergenti* and lesions of cutaneous leishmaniasis in an emerging focus of Morocco. *Parasit Vectors* 2013; **6**: 217.

[28] Es-Sette N, Ajaoud M, Laamrani-Idrissi A, Mellouki F, Lemrani M. Molecular detection and identification of *Leishmania* infection in naturally infected sand flies in a focus of cutaneous leishmaniasis in Northern Morocco. *Parasit Vectors* 2014; **7**: 305.

[29] Tabbabi A, Bousslimi N, Rhim A, Aoun K, Bouratbine A. First report on natural infection of *Phlebotomus sergenti* with *Leishmania* promastigotes in the cutaneous leishmaniasis focus in Southeastern Tunisia. *Am J Trop Med Hyg* 2011; **85**: 646-7.

[30] Gebre-Michael T, Balkew M, Ali A, Ludovisi A, Gramiccia M. The isolation of *Leishmania tropica* and *L. aethiopica* from *Phlebotomus* (Paraphlebotomus) species (Diptera: Psychodidae) in the Awash Valley, Northeastern Ethiopia. *Trans R Soc Trop Med Hyg* 2004; **98**: 64-70.

[31] Sharifi I, Nakhaei N, Aflatoonian M, Parizi MH, Fekri A, Safizadeh H, et al. Cutaneous leishmaniasis in bam: a comparative evaluation of pre- and post-earthquake years (1999-2008). *Iran J Public Health* 2011; **40**(2): 49-56.

[32] Svobodová M, Votýpka J, Nicolas L, Volf P. *Leishmania tropica* in the black rat (*Rattus rattus*): persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti*. *Microbes Infect* 2003; **5**: 361-4.

[33] Sádlová J, Hajmová M, Volf P. *Phlebotomus* (*Adlerius*) *halepensis* vector competence for *Leishmania major* and *Le. tropica*. *Med Vet Entomol* 2003; **17**: 244-50.

[34] Dvorak V, Votypka J, Aytekin AM, Alten B, Volf P. Intraspecific variability of natural populations of *Phlebotomus sergenti*, the main vector of *Leishmania tropica*. *J Vector Ecol* 2011; **36**(Suppl 1): S49-57.