Molecular detection of *Leishmania* parasites and host blood meal identification in wild sand flies from a new endemic rural region, south of Iran

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Zoonotic Cutaneous Leishmaniosis (ZCL) remains the most crucial vector-borne public health disease particularly in endemic rural parts of Iran. The main aim of this study is to identify wild sand flies (Diptera: Psychodidae), determine their infection rate, and differentiate their host blood meal sources using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Sand fly populations were caught with sticky paper traps from 10 different villages in the county of Darab, Fars province, southern Iran. Following their species identification, they were used in one step PCR to determine their infection with *Leishmania* spp. parasites. They were then subjected to PCR-RFLP protocol to identify and differentiate their blood meal sources. Two genera of *Phlebotomus* and *Sergentomyia* comprising 13 species of sand flies were identified in this region. From a total of 150 parous female sand flies, encompassing 4 different medically important species, 7 specimens (4.7%) including 6 *Phlebotomus papatasi* and 1 *Phlebotomus bergeroti* were infected with *Leishmania major*. Molecular data indicated that about 32% of female sand flies fed on man, while nearly 43% fed on rodent and canine hosts. Molecular detection is an efficient way of differentiating the source of blood meals in female sand flies feeding on different vertebrate hosts. It is suggested that *P. papatasi* is not highly anthropophagic and appears to be an opportunistic feeder on man. This species is, however, the primary vector of ZCL in this region.

Keywords: Blood meal identification, Phlebotomine sand flies, PCR-RFLP, *Leishmania* spp, Iran

Introduction

Leishmaniases are vector-borne parasitic diseases caused by the heteroxenous (i.e. living in dissimilar hosts) protozoa from the kinetoplastid genus *Leishmania* (Trypanosomatida: Trypansomatidae). This dixenous genus together with the monoxenous genus *Crithidia* forms the subfamily Leishmaniinae.[1] They are endemic in many countries (>98 countries) on 5 continents threatening 350 million people with 12 million cases of infection.[1] Based on the site of infection, viscera or skin, in mammalian tissues, the intracellular *Leishmania* parasites produce two broad clinical forms of the disease known as visceral and cutaneous leishmaniasis (CL). Human CL with a global incidence of about 0.7–1.2 million cases is the most prevalent form of disease.[2] It is a disfiguring skin lesion caused by about 15 different species of the genus *Leishmania*.

In the Old World, *Leishmania* is transmissible by the bite of hematophagous infectious female phlebotomine sand flies (Diptera: Psychodidae, subfamily Phlebotominae) feeding alternately on an infected reservoir mammal and a susceptible human host.[3] The dispersal and geographical distribution of *Leishmania* thus follows the spatio-temporal overlap of its hosts and vectors together. An intimate specific relationship has also been shown between certain sand flies and *Leishmania* species, for instance, *Leishmania major* and *Phlebotomus papatasi*. A long evolutionary history of interactions between *Leishmania* and sand flies has thus led to a strong sympatric distribution.[1]

Based on the clinical signs and symptoms, there are two different forms of human CL in Iran. A wet, rural, and rodent-based form known as zoonotic CL (ZCL) and caused by *L. major* is commonly transmitted by *P.*

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Figure 1 Sketch map of Iran showing the locations of 10 study villages (The numbers of 1–10 correspond to Arabghanbari, Sangcharak, Dolatabad, Banuj, Behroozabad, Talbargah, Keirabad, Ghalebiaban, Nasirabad, Fadami, respectively.) in the County of Darab, Fars province, southern Iran. (Source: Author)

papatasi;[4] whereas a dry, urban, and human- (or canine-) based form called anthroponotic CL (ACL) developed by *L. tropica* is vectored by *P. sergenti*.[5] The ACL type is less abundant. It is not immunologically protected from the rural type, which means patients with ACL may be re-infected with *L. major* once they get exposed to it.

Female sand flies are the wild vectors of *Leishmania* parasites in Fars province of Iran.[6] The main vector of *L. major*, the sand fly *P. papatasi*, is highly associated with known colonies of rodent reservoir hosts dispersed in adjacent ecologic often intensely irrigated areas of human dwellings.[7] The association of agricultural and rural development practices together with the spread of human populations into the habitats of the local blood sucking vector and the rodents that act as reservoir hosts, have led to increase in ZCL cases in recent decades.[8]

Sand fly females need to engorge on vertebrate host blood meal to supplement the essential proteins for egg production. Certain sand fly species may be attracted to a particular animal host species or group, while most others tend to be generalists rather than specialists in their host specificity. This is evident in *P. papatasi*, a generalist vector of *L. major* in the Old World, indicating that host selection is merely a matter of presence rather than preference.[9] Understanding the bloodsucking pattern of these sand flies, which feed on a wide range of mammalian hosts, including man, rodents, domestic livestock, dogs and birds, is of immense eco-epidemiological significance, since any data on their host blood meal sources could help identify the potential reservoir hosts and provide basic epidemiological information for vector control programs.[10]

Since the rate of sand fly infection with *Leishmania* is generally negligible (0.01–1%) even in endemic areas,[11] the use of alternative molecular methods for the detection and identification of *Leishmania* species in sand flies is highly suitable. The application of the kinetoplastid DNA polymerase chain reaction (PCR) diagnostic assay was reported to be very sensitive (98.7%) for *Leishmania* parasites.[12] This method is sensitive and needs slight effort and thus will be a powerful tool for more research on sand flies and the relationships between *Leishmania* species and their vectors. The PCR-restriction fragment length polymorphism (PCR-RFLP) is useful for blood meal identification to trace the source of host blood meal species. It uses restriction enzymes which cleave DNA at specific sequences.[13,14]

The main aims of the present study are thus to identify the randomly distributed wild sand fly fauna in an area covered by 10 villages, determine *Leishmania* infection rate in phlebotomine sand flies, and detect the potential sources of their vertebrate blood meal hosts using the PCR-RFLP technique in the endemic rural region of Darab, Fars province, south of Iran.

Materials and methods

Study area

The county town of Darab (54°30′E, 28°50′N) lies in Fars province, south of Iran. Its area is about 7500 km^2 located approximately 1180 meters above sea level. In 2012 census, it had a population of 196251 people with 44.7% living in urban areas. Most people are involved in agricultural practices. The vast areas of pastoral land covers are interspersed with significant arable patches. The weather in Darab varies between frigid winters and scorching dry summers. The temperature ranges from –4 to 46 °C and the average annual precipitation is about 160 mm.

Sand fly collections

Based on the variety of climate and topography, a representative sample of 10 villages including Arabghanbari, Behroozabad, Banuj, Dolatabad, Fadami, Ghalebiaban,

Kheirabad, Nasirabad, Sangcharak, and Talbargah were randomly selected for sand fly collections from May to September 2012 (Fig. 1). Sticky trap using sheets of white A4 paper soaked in castor oil is the most common method for sampling of sand flies. In each village, 30 sticky traps were set up for either indoor (inside houses and domestic animal shelters) or outdoor (outside settlements mostly near entries to rodent burrows) resting sites at each sampling trial. Sand flies were separated with fine-pointed needles from the papers rinsed in acetone, and preserved in 70% ethanol. They were identified using valid taxonomic keys. All sand flies were dissected under a loop microscope. Heads and last abdominal segments were placed in Puri's clearing medium on a microscope slide for 24 h and were dry mounted.[15] The remaining body segments of female sand flies were used for DNA extraction and subsequent PCR processing.

DNA extraction

DNA was extracted using the phenol-chloroform method as described elsewhere.[15] Body segments from each sand fly were briefly and mechanically disrupted with 100 µl lysis buffer; 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 1% Tween 20; bearing 3.5 µl of a proteinase K solution (16 µg/ml) in a 1.5 ml microtube during 24 h at 37 °C temperature. This mixture was then added to 200 µl of a phenol: chloroform: isoamyl alcohol solution (25:24:1 by volume) and incubated at room temperature for 10 min and then centrifuged. The DNA was subsequently precipitated with 400 µl cold pure ethanol and suspended in 50 µl ddH_2O and stored at –20 °C until the DNA was amplified by PCR.

Detection of *Leishmania* by PCR amplification

PCR assay was conducted to amplify the variable area of the minicircle kDNA of any *Leishmania* present in the sand fly. The forward and reverse primers of Lin17 (TTT GAA CGG GAT TTC TG) and LinR4 (GCG GTT GGT GTA AAA TAG GG) were used. PCR was carried out in a total volume of 25 µl reaction mixture containing 0.5 µl of deoxynucleoside triphosphate, 1.5 µl of $MgCl_2$, 0.5 µl of DNA *Taq* polymerase (Cinagen, Tehran, Iran), 1.5 µl of each primers, 2.5 µl PCR buffer (Boehringer Mannheim, Germany), and 5 µl of DNA extract in 12 µl distilled water. This mixture was then incubated in a thermocycler (Eppendorf AG; Hamburg, Germany) set to run at 94 °C for 5 min, 30 cycles each comprising 30 s at 94 °C, 30 s at 52 °C, 1 min at 72 °C, and a final extension at 72 °C for 10 min.

In all PCR assays, DNAs from male sand flies were used as negative controls. Reference strains of *Leishmania infantum* (MCAN/IR/96/Lon49) 720 bp, *L. tropica* (MHOM/IR/89/ARD2) 760 bp, and *L. major* (MHOM/IR/54/LV39) 650 bp; were used as positive controls.[16] These reference strains were acquired from the Medical Parasitology Laboratory, Faculty of Medicine, Shiraz University of Medical Sciences.

For electrophoresis, PCR products were run in 1.5% agarose gel. The resulting DNA bands were stained with 1% ethidium bromide and visualized on an UV transilluminator. The size of each sample band was estimated by comparison with the size of reference strains from *L. infantum, L. tropica* and *L. major* parasites.

Host blood meal resources in sand flies by PCR-RFLP

To evaluate the sources of host blood meal, 56 engorged female sand flies were selected for DNA extraction and PCR amplification. Blood meals were identified using specific primers of cytB forward (5′ CCA TCC AAC ATC TCA GCA TGA TGA AA 3′) and cytB reverse (5′ CCC CTC AGA ATG ATA TTT GTC CTC A 3′) that amplify a 358 bp part of cytochrome b gene in the mitochondrial DNA (mtDNA) of mammalian hosts. This segment is digested by *Xho*I enzyme in human DNA (215 and 143 bp), but it has no restriction site in the DNA from other mammalians. To identify animal host blood meals, a second region of the mtDNA was amplified using forward and reverse primers of UNFOR403 (5′ TGA GGA CAA ATA TCA TTC TGA GG 3′), and UNREV1025 (5′ GGT TGT CCT CCA ATT CAT GTT A 3′), respectively. These primers amplified a 623 bp region of the cytB gene of mammalian mtDNA. This fragment is digested by *Hae*III enzyme in DNA from mammalian hosts rather than humans. Following digestion, *Hae*III enzyme produces two 345 and 304 bp segments from cow DNA, two 552 and 70 bp segments from canine DNA, and two 453 and 170 bp segments from goat DNA.[13]

In PCR-RFLP assay, human or cow blood are used as positive controls and a male or unfed female sand fly as negative controls. The mixture was prepared in a total volume of 25 µl containing 0.5 µl of dNTPs, 0.5 µl of $MgCl_2$, 0.5 µl of DNA *Taq* polymerase (Cinagen, Tehran, Iran), 1 µl of each primers, 2.5 µl of PCR buffer (Boehringer Mannheim, Mannheim, Germany), and 2.5 µl of DNA extract in 16 µl ddH_2O. This mixture was incubated in a thermocycler as before set to run for 3.5 min at 95 °C, followed by 36 cycles each consisting of 30 s at 95 °C, 50 s at 58 °C, and 40 s at 72 °C and a final extension at 72 °C for 5 min.

Since *Hae*III enzyme has no restriction site on the 623 bp region in humans but with various specific sites in mtDNA of other mammalian hosts, it was used to identify blood meal resources in sand flies. Digestion of PCR products was performed in 25 µl of a solution containing 15 µl of PCR extract mixed with 2.5 µl of enzyme buffers and 5 units of the restriction enzyme overlaid with two drops of mineral oil. This mixture was then incubated overnight at 37 °C. The digestion product was mixed with loading buffer and then put onto a 2.5% agarose gel. This was subjected to electrophoresis. Gels were stained with ethidium bromide and the RFLP profiles were visualized under ultraviolet light.

Results

The geographical distribution of different sand fly species in Darab County, Fars province of south Iran demonstrates

Table 1 Geographical distribution of different sand fly species in Darab County, Fars province, Iran, 2012

Sand fly species/Village No. (%)		Sangcharak	Behroozabad	Dowlatabad	Arabghanbari	Kheirabad	Ghalebiaban	Nasirabad	Fadami	Talbar-gah	Banuj	Sum (%)
Phlebotomus	*papatasi*	409 (8.15)	401 (7.99)	164 (3.27)	110 (2.19)	130 (2.59)	236 (4.70)	80 (1.59)	306 (6.10)	64 (1.27)	570 (11.36)	2470 (49.21)
	alexandri	433 (8.62)	253 (5.04)	0 (0)	0 (0)	22 (0.44)	24 (0.48)	28 (0.56)	6 (0.12)	12 (0.24)	108 (2.15)	886 (17.65)
	sergenti	43 (0.85)	48 (0.96)	0 (0)	2 (0.04)	0 (0)	2 (0.04)	0 (0)	0 (0)	0 (0)	6 (0.12)	101 (2.01)
	bergeroti	3 (0.06)	3 (0.06)	0 (0)	8 (0.16)	2 (0.03)	2 (0.03)	0 (0)	56 (1.12)	0 (0)	8 (0.16)	82 (1.63)
	elenori	0 (0)	3 (0.06)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.06)
Sergentomyia	*dentata*	232 (4.62)	70 (1.39)	28 (0.56)	46 (0.92)	92 (1.83)	52 (1.03)	22 (0.44)	28 (0.56)	0 (0)	56 (1.12)	626 (12.47)
	tiberiadis	0 (0)	3 (0.06)	0 (0)	0 (0)	0 (0)	24 (0.48)	0 (0)	20 (0.4)	0 (0)	0 (0)	47 (0.94)
	clydei	14 (0.28)	10 (0.20)	0 (0)	0 (0)	0 (0)	44 (0.88)	0 (0)	56 (1.12)	0 (0)	0 (0)	124 (2.47)
	theodori	44 (0.88)	11 (0.22)	18 (0.36)	18 (0.36)	88 (1.75)	42 (0.84)	14 (0.28)	88 (1.75)	0 (0)	0 (0)	323 (6.44)
	sintoni	23 (0.46)	8 (0.16)	0 (0)	0 (0)	24 (0.48)	0 (0)	20 (0.4)	74 (1.47)	16 (0.32)	54 (1.07)	219 (4.36)
	squamipleuris	9 (0.18)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (0.18)
	baghdadis	16 (0.32)	0 (0)	10 (0.2)	10 (0.2)	20 (0.4)	0 (0)	0 (0)	0 (0)	6 (0.12)	6 (0.12)	68 (1.36)
	antennata	3 (0.06)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	58 (1.15)	0 (0)	0 (0)	61 (1.21)
Total (%)		1229 (24.49)	810 (16.14)	220 (4.38)	194 (3.86)	378 (7.53)	426 (8.49)	164 (3.27)	692 (13.79)	98 (1.95)	808 (16.10)	5019 (100)

that almost half (49.2%) of all the trapped species belonged to *Phlebotomus papatasi*, the main vector of ZCL in Iran, followed by *P. alexandri* and *Sergentomyia dentata* being the second and third most abundant species, respectively (Table 1). Only two genera of *Phlebotomus* and *Sergentomyia* comprising 13 species of sand flies were identified in this region. The village of Sangcharak contained about a quarter (24.5%) of all the sand flies. In contrast, the village of Talbargah had the lowest (1.95%) abundance of sand flies.

Leishmania species detection in sand flies

A total of 150 parous female sand flies comprising 118 *Phlebotomus papatasi*, 12 *P. alexandri*, 8 *P. sergenti*, and 12 *P. bergeroti* were examined for *Leishmania* DNA. From these, only 7 specimens (4.7%) including six *P. papatasi* and one *P. bergeroti* were shown to be positive with the *Leishmania major* parasite DNA. The former sand flies were captured from both indoor and outdoor sites at Sangcharak and Behroozabad villages during August and September months. In addition, positively identified *P. bergeroti* sand flies were trapped from outdoor sites at Fadami village in June. The gel electrophoresis of PCR products indicated a single band equal to 650 bp for the positively identified sand flies in line with the obtained standard strain band of *L. major* (Fig. 2).

Sand fly blood meal identification

The presence of host blood meal within sand fly abdomen was initially confirmed by visual inspection. Overall, 100 blood fed female sand flies were thus selected which included the 10 species of *P. papatasi*, *P. alexandri*, *P. sergenti, P. bergeroti, Sergentomyia dentata, S. tiberiadis, S. clydei, S. sintoni, S. squamipleuris,* and *S. baghdadis*. Since there was no yield in PCR products of negative control, this implied that only host DNA was amplified. That is sand fly DNA is likely to be present but cannot be amplified and subsequently be detected by the mammal-specific PCR.

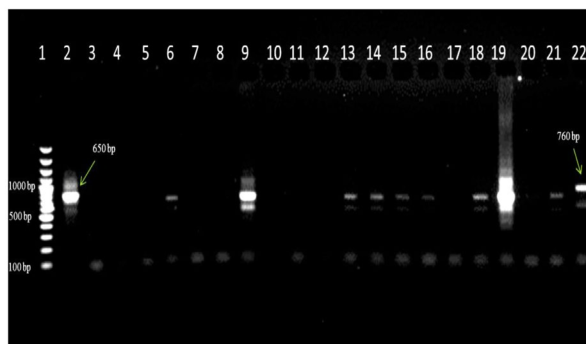

Figure 2 Gel electrophoresis of PCR products of sand flies from Darab County, Fars province, Iran, in 2012. The bands correspond to the molecular weight marker (Lane 1), reference strains of *Leishmania major* (Lanes 2 and 9), reference strains of *Leishmania tropica* (Lane 22), negative control (Lanes 3), uninfected samples (Lanes 4, 5, 7, 8, 10–12, 17 and 20), infected sand flies (Lanes 6, 13–16, 18 and 21).

Table 2 Host blood meal sources of naturally fed sand flies from vertebrate animals in Darab County, Fars province, Iran, 2012

Sand fly species	Sand flies host blood meal sources							
	Mammalians	Non-mammalians	Total	Human	Rodent & Dog	Human & Cow	Cow & Dog or Rodent	Total
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Phlebotomus papatasi	42	34	76	12 (28.6)	18 (42.9)	9 (21.4)	3 (7.1)	42
P. bergeroti	0	1	1	0	0	0	0	0
P. alexandri	5	0	5	3 (60)	2 (40)	0	0	5
P. sergenti	1	0	1	1 (100)	0	0	0	1
Sergentomyia clydei	2	2	4	0	2 (100)	0	0	2
S. squamipleuris	0	4	4	0	0	0	0	0
S. sintoni	2	2	4	1 (50)	0	1 (50)	0	2
S. dentata	2	0	2	1 (50)	0	1 (50)	0	2
S. tiberiadis	1	0	1	0	1 (100)	0	0	1
S. baghdadis	1	1	2	0	1 (100)	0	0	1
Total	56	44	100	18 (32.1)	24 (42.9)	11 (19.6)	3 (5.4)	56 (100)

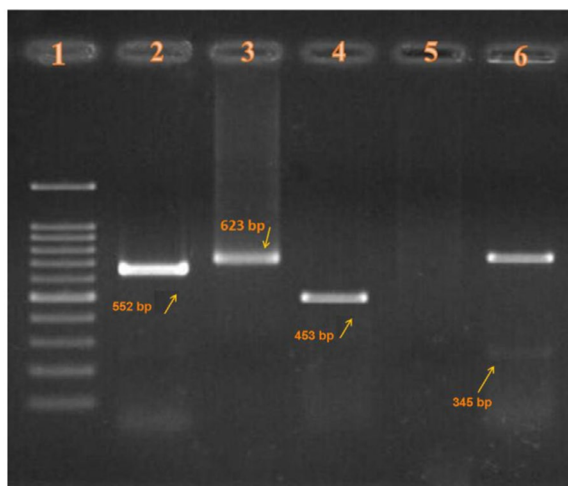

Figure 3 Electrophoresis of mitochondrial DNA cytochrome b gene fragments from blood meals of sand flies digested with *Hae*III. Lane 1, molecular weight marker (100 bp, Cinnagen, Iran); Lane 2, dog blood; Lane 3, human blood; Lane 4, goat blood; Lanes 5, male sand flies as non-blood meal feeding insect; Lane 6, mixture of human (upper band) and cow (lower band) blood.

The primary data on PCR indicated mammalian and non-mammalian blood meals were present in 56 and 44 female sand fly specimens, respectively (Table 2). Further analysis by PCR-RFLP assay on the former set of specimens revealed that rodent/dog and humans accounted for 43 and 32% of blood meal sources in the sample of sand flies (Fig. 3). It was also asserted that the remaining quarter belonged to mixed blood meals from human and cow, or cow and dog or rodent sources.

Discussion

In the old world, the main vector of *Leishmania major* is the sand fly *Phlebotomus papatasi,* although other sand fly species, such as *P. salehi*, are likely to be incriminated.[8] The vector, *P. papatasi*, establishes the major transmission link between reservoir and human hosts.[7,17] Most rural areas of Fars province, south of Iran, are endemic for the ZCL disease.[3,4,18] Three different species of *L. major, L. turanica* and *L. gerbilli* have been detected from *P. papatasi* sand flies collected near rodent burrows and animal shelters in Isfahan and Golestan provinces of Iran.[19] There is, however, a low diversity of *Leishmania* parasites in sand flies, as rodent reservoir host species could change abruptly from one province to the next.[20] Only *L. major* was detected from *P. papatasi* in the present study which is in line with another study from the central Iranian province of Isfahan.[21] On the other hand, the infection of *Phlebotomus bergeroti* sand fly with *L. major* parasite was first reported recently in Iran. In the current investigation, strictly speaking, the infection level in *P. bergeroti* (1:12; 8.33%) predominated that in *P. papatasi* (6:118; 5.08%) species, as it is reported from other studies carried out in Fars and Isfahan provinces.[20] The high density of *P. papatasi* either in indoor resting places or outdoors such as rodent burrows implies that this species plays a significant role as the principle vector in this region.[6,7,22]

According to Killick-Kendrick,[23] the main vector incrimination criteria are the demonstration that the sand fly is anthropophilic and/or anthropophagic. The second criterion is the frequent isolation and identification of the same species of *Leishmania* from both the sand fly and the patient. The sand fly normally feeding on the vertebrate reservoir hosts should frequently be found in areas where the parasite and the disease it causes are available. It could transmit the *Leishmania* by bite and sustain its development over several generations. Merely abundance of a sand fly species in a place is not sufficient evidence to incriminate it as a vector. As indicated above, although other species of sand flies such as *P. caucasicus, P. mongolensis,* and *P. ansarii* have been implicated as possible vectors, only *P. papatasi* has so far been incriminated to be a general vector of *L. major* parasite in Iran.[19]

The identification of the blood meals of hematophagous insects were until recently conducted using serological techniques such as the precipitin test, latex agglutination test, and the enzyme-linked immune-sorbent assays

(ELISA) which are regarded to be time-wasting and with low level of sensitivity. Nowadays, molecular methods are used as potential alternatives. As in other previous studies,[13] the origin of blood meals in sand flies were determined by analyzing the PCR-RFLP cytochrome b gene in the present study.

Apart from the major role played by *P. papatasi* sand flies in host blood feeding (76%), the current data revealed that non-mammalian host blood meals were taken up by one *P. bergeroti* and nine *Sergentomyia* spp. On the other hand, mammalian blood meals were traced back in one *P. sergenti*, five *P. alexandri*, and eight *Sergentomyia* spp. In contrast to other early studies in which *P. papatasi* was introduced as a highly anthropophilic vector in Isfahan province,[21] the present data indicated that <30% of *P. papatasi* population fed on humans. It is thus concluded that in this study area *P. papatasi* is not highly anthropophilic. It appears to be an opportunistic feeder depending on the presence of a wide range of natural hosts, encompassing mammalians and non-mammalians, to safeguard its reproduction and survival in this region. Moreover, since the anthropophilic rate of *P. papatasi* in not high in this region, it is suggested that the potential vector capacity of other phlebotomine sand flies be explored in future.

Finally, since Fars province is endemic to a number of vector-borne diseases,[24–27] collection of data on sand flies is useful in planning and evaluation of intervention activities to reduce disease incidence. The identification of host blood meals is conducive to the elucidation of vector-borne pathogen transmission cycles and the risk of disease could be implicated in this region.

Conflicts of interest

No conflict or competing financial interests exists.

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References

1 Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS Negl Trop Dis. 2016;10(3):e0004349.

2 Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671.

3 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;111:1821–1826.

4 Azizi K, Badzohreh A, Sarkari B, Fakoorziba MR, Kalantari M, Moemenbellah-Fard MD, et al. Nested polymerase chain reaction and sequenced-based detection of *Leishmania* infection of sand flies in recently emerged endemic focus of zoonotic cutaneous leishmaniasis, southern Iran. Iran J Med Sci. 2013;38(2):156–162.

5 Moemenbellah-Fard MD, Ahmadyousefi-Sarhadi M, Azizi K, Fakoorziba MR, Kalantari M, Amin M. Faunal identification and frequency distribution of wild sand flies infected with *Leishmania* tropica. Asian Pac J Trop Dis. 2015;5(10):792–797.

6 Azizi K, Parvinjahromi H, Moemenbellah-Fard MD, Shahriari B, Fakoorziba MR. Faunal distribution and seasonal bio-ecology of naturally infected sand flies in a new endemic zoonotic cutaneous leishmaniasis focus of southern Iran. J Arthropod-Borne Dis. 2016;10(4):560–568.

7 Azizi K, Rassi Y, Moemenbellah-Fard MD. PCR-based detection of *Leishmania* major kDNA within naturally infected *Phlebotomus papatasi* in southern Iran. Trans R Soc Trop Med Hyg. 2010;104:440–442.

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 Sales KGS, Costa PL, de Morais RCS, Otranto D, Brandão-Filho SP, Cavalcanti MP, et al. Identification of phlebotomine sand fly blood meals by real-time PCR. Parasit Vectors. 2015;8:127–235.

10 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):844–850.

11 Kato H, Uezato H, Gomez EA, Terayama Y, Calvopiña M, Iwata H, et al. Establishment of a mass screening method of sandfly vectors for *Leishmania* infection by molecular biological methods. Am J Trop Med Hyg. 2007;77:324–329.

12 Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous Leishmaniasis. J Clin Microbiol. 2006;44:1435–1439.

13 Maleki-Ravasan N, Oshaghi M, Javadian E, Rassi Y, Sadraei J, Mohtarami F. Blood meal identification in field captured sand flies: comparison of PCR-RFLP and ELISA assays. Iran J Arthropod-Borne Dis. 2009;3:8–18.

14 kent RJ. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol Ecol Resour. 2009;9:4–18.

15 Azizi K, Rassi Y, Javadian E, Motazedian M, Rafizadeh S, Yaghoobi-Ershadi M, et al. *Phlebotomus* (*Paraphlebotomus*) *alexandri*: a probable vector of *Leishmania* infantum in Iran. Ann Trop Med Parasitol. 2006;100:63–68.

16 Azizi K, Soltani A, Alipour H. Molecular detection of *Leishmania* isolated from cutaneous leishmaniasis patients in Jask county, Hormozgan province, southern Iran, 2008. Asian Pac J Trop Med. 2012;5(7):514–517.

17 Hamarsheh O. Distribution of *Leishmania* major zymodemes in relation to populations of *Phlebotomus papatasi* sand flies. Parasit Vectors. 2011;4:1–6.

18 Razmjou S, Hejazy H, Motazedian MH, Baghaei M, Emamy M, Kalantary M. A new focus of zoonotic cutaneous leishmaniasis in Shiraz. Iran Trans R Soc Trop Med Hyg. 2009;103:727–730.

19 Parvizi P, Ready P. Nested PCRs and sequencing of nuclear ITS-rDNA fragments detect three *Leishmania* species of gerbils in sand flies from Iranian foci of zoonotic cutaneous leishmaniasis. Trop Med Int Health. 2008;13:1159–1171.

20 Parvizi P, Alaeenovin E, Kazerooni P, Ready P. Low diversity of *Leishmania* parasites in sand flies and the absence of the great gerbil in foci of zoonotic cutaneous leishmaniasis in Fars province, southern Iran. Trans R Soc Trop Med Hyg. 2013;107:356–362.

21 Parvizi P, Akhoundi M, Mirzaei H. Distribution, fauna and seasonal variation of sand flies: simultaneous detection of nuclear internal transcribed spacer ribosomal DNA gene of *Leishmania* major in *Rhombomys opimus* and *Phlebotomus papatasi*, in Natanz district in central part of Iran. Iran Biomed J. 2012;16:113–120.

22 Yaghoobi-Ershadi M. Phlebotomine sand flies (Diptera: Psychodidae) in Iran and their role on *Leishmania* transmission. J Arthropod Borne Dis. 2012;6:1–17.
23 killick-kendrick R. Phlebotomine vectors of the leishmaniases: a review. Med Vet Entomol. 1990;4:1–24.
24 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;106:358–365.
25 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):2681–2684.
26 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. 2016;40(1):169–175.
27 Khosravani M, Moemenbellah-Fard MD, Sharafi M, Rafat-Panah A. Epidemiologic profile of oriental sore caused by *Leishmania* parasites in a new endemic focus of cutaneous leishmaniasis, southern Iran. J Parasit Dis. 2016;40(3):1077–1081.