

## Original Article

# Faunal Distribution and Seasonal Bio-Ecology of Naturally Infected Sand Flies in a New Endemic Zoonotic Cutaneous Leishmaniasis Focus of Southern Iran

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## Abstract

**Background:** Cutaneous leishmaniasis is a major health problem in Iran in spite of implementation of control program. This infectious disease caused morbidity in less than 27000 people in 2010. This study was set to determine some ecological aspects of sand flies in Fasa district, Fars Province, southern Iran during 2011–2012.

**Methods:** A total of 4792 sand flies were captured by means of sticky paper and CDC miniature light traps in 10 selected villages from the beginning to the end of the active season, from which 1115 specimens were captured for abundance study and 3677 specimens captured for monitoring monthly activities in Fasa. After species identification, extracted DNA was processed for detection of *Leishmania* parasite infection in sand flies.

**Results:** Twelve species (6 *Phlebotomus*, 6 *Sergentomyia*) were identified. The most common sand fly was *P. papatasi* (82.4%) which represented 86.6% of sand flies from indoors and 82.7% from outdoors. The monthly activity of the species extended from April to the end of November. There were two peaks in the density curve of this species, one in June and the second in September. Natural infection to *L. major* was detected in *P. papatasi* (25 out of 130 sand flies, 19.2%).

**Conclusion:** *Phlebotomus papatasi* is considered as a main vector of zoonotic cutaneous leishmaniasis in Fasa, Fars Province, south of Iran.

**Keywords:** Sand flies, *Leishmania*, Vector, *Phlebotomus*, Iran

## Introduction

*Leishmania* parasites can induce a range of clinical manifestations exhibited from self-healing localized dermal lesions to chronic non-healing diffuse muco-cutaneous forms (Davami et al. 2010, Shirian et al. 2011). Human cutaneous leishmaniasis (CL) remains a pressing public health problem in many countries of the Eastern Mediterranean region, including Iran. It is the most important and widely known malady after malaria in Iran (Moemenbellah-Fard et al. 2012). It is a group of clinically complex sand fly-borne skin infections caused by different species of the blood flagellate protozoan parasites in the ge-

nus *Leishmania* (Kinetoplastida: Trypanosomatidae). About 0.7–1.3 million cases of CL occur in 88 countries annually (Desjeux 2001). CL appears to be a major health concern, which, despite its notifiable status, necessitates urgent action to contain its increasingly reported cases (Abai et al. 2007, Azizi et al. 2010, 2012a-d, Fakoorziba et al. 2011). More than a quarter of all CL cases (n= 26,824) registered in 2010 within the Eastern Mediterranean region were Iranians (Postigo 2010).

This skin lesion is reported from many old world and new world countries with over 90% of cases occurring only in seven coun-

tries including Iran, Afghanistan, Saudi Arabia, Syria, Brazil, Nepal and Peru (Desjeux 2004). The clinical signs and symptoms of CL in humans are in two forms: dry or anthroponotic (Anthroponotic Cutaneous Leishmaniasis or ACL) and wet or zoonotic (Zoonotic Cutaneous Leishmaniasis or ZCL) forms.

Leishmaniasis includes parasitic infections caused by the interaction of a wide variety of vectors and reservoirs spread throughout the world except Australia (Ashford 2000). *Leishmania*-like parasites were shown in kangaroos of Australia (Rose et al. 2004). Leishmaniasis is mainly known as a neglected disease in tropical regions.

Hematophagous females of some sand flies (Diptera: Psychodidae, subfamily Phlebotominae) are the natural vectors of *Leishmania* parasites in Fars Province (Oshaghi et al. 2010, Azizi et al. 2013). The sand fly, *P. papatasi*, which is the main incriminated vector of *L. major*, is mostly associated with colonies of different rodent reservoir hosts distributed in various regions of Iran (Moemenbellah-Fard et al. 2003, Azizi et al. 2011, 2012c, Parvizi et al. 2013, Akhoundi et al. 2013, Davami et al. 2014).

Human CL is endemic in more than half of the 31 Iranian provinces. The majority of CL cases in Iran are caused by *L. major* (Akhoundi et al. 2013). ZCL is widespread in the central, southern, eastern and western provinces of Iran.

The county town of Fasa is one of the most important endemic foci of ZCL in Fars Province, southern Iran, with 1088 cases in 2007 and 185 cases in 2011. The main aim of this investigation was to capture and identify the vectors of ZCL in this focus using nested PCR. Therefore, faunal distribution and seasonal activity of naturally infected sand flies were investigated in this new endemic ZCL focus of southern Iran. To the best of our knowledge, this is the first study on the infection of sand flies with *Leishmania* parasites in this county of southern Iran.

## Materials and Methods

### Study Area

This investigation was conducted in Fasa county (29°24'N, 54°15'E), Fars Province, southern Iran (Fig. 1). It is about 4,000 km<sup>2</sup> in area and 1,370-meter altitude above the sea level. The total population of this county town was about 220,000 people in 2010. The climatic condition is very hot in summer and cold dry during winter. The main activities of the people are agriculture and farming.

### Sand Fly Collection

Sand flies were collected from ten villages, selected based on history of CL prevalence, differential topography, and the mere presence of vectors and reservoirs of infection. Sand flies were caught biweekly in two villages (Fedeshkooyeh and Miandeh) from fixed sites indoors (bedrooms, sitting rooms, toilets and stables) and outdoors (rodent burrows), using 30 sticky traps (castor oil-coated white papers measuring 20 cm×30 cm) from the beginning to the end of the active season (May-September) to determine sand fly monthly activity. Each trap set on one night was taken as a "trap-night" and there were 120 "trap-nights"/month/village. For vector faunal study, eight villages at different points of the county town of Fasa were selected randomly. Sand flies were collected three times during the active season by using sticky paper and CDC traps. Trapped sand flies were removed from sticky papers with needles, washed in absolute acetone and stored in 70% ethanol. For species identification, sand flies were mounted in Puri's medium (Smart 1965). They were identified using the criteria set in the keys of Theodor and Mesghali (1964), and then mounted and segregated by sex. The middle body segments (apart from heads and last abdominal segments) of some unfed parous female sand flies with the mid-guts were kept in 70% ethanol for DNA extraction and subsequent PCR processing.

### DNA Extraction

Each female sand fly was transferred to a microtube for DNA extraction as described elsewhere (Azizi et al. 2008). The sand fly sample with DNA was added to a microtube containing 100 µl lysis buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% (v/v) Tween 20] and 12 µl of a proteinase K solution (20 µg/ml), in a 1.5 ml tube (Motazedian et al. 2002). The tube was incubated for 24 h at 37 °C before 200 µl of a phenol:chloroform: isoamyl alcohol mixture (25:24:1, by vol.) was added. It was stored at room temperature for 5 min then it was centrifuged at 15,000 rpm for 15 min. The supernatant solution with DNA was then taken with sampler and 200 µl cold absolute ethanol was added. It was centrifuged at 15,000 rpm for 5 min at 4 °C, then it was evicted on a floated solution in the tube and then stored at 37 °C and 50 µl double distilled water was added and then stored at -20 °C, until it could be tested for *Leishmania* kDNA.

### PCR amplification

The PCR used to amplify the variable area of the minicircle kinetoplastic DNA of any *Leishmania* in sand fly is described elsewhere (Aransay et al. 2000). The primers were 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. They were designed within the conserved area of the minicircle kinetoplast containing the conserved sequence blocks 3 and 2. Each 25 µl reaction mixture contained 0.5 µM of each deoxynucleoside triphosphate, 1 mM MgCl<sub>2</sub>, 1 unit *Taq.* polymerase (CinnaGen, Tehran, Iran), 0.5 µM CSB1XR, 0.5 µM CSB2XF primers, 5 µl DNA extract, 2.5 µl PCR buffer and 0.3 µl DNA polymerase. PCR reaction mixture was set at 94 °C for 5 min, followed by 30 cycles, each of 30 s at 94 °C, 1

min at 55 °C and 1.5 min at 72 °C, and then a final extension at 72 °C for 5 min in a thermocycler (Eppendorf AG: Humburg, Germany). For the second round of PCR, only 1 µM of each of 13Z and LiR primers were used. One µl of the first round products with 1/9 dilution (by vol.) were used as templates for the second round of PCR. Five µl of final products were subjected to electrophoresis on 1.5% (V/V) agar gel stained with ethidium bromide and visualized by UV transillumination. The size of each band was estimated by comparison with the size of reference strains of *L. infantum* (MCAN/IR/96/LON49), *L. tropica* (MHOM/IR/89/ARD2) and *L. major* (MHOM/IR/54/LV39). A band of 560 bp indicated that *L. major* kDNA was present (Azizi et al. 2012d).

### Results

A total of 4792 sand flies were captured by means sticky paper and CDC miniature light traps in 10 selected villages, from which 1115 specimens were counted for abundance study and 3677 specimens were monitored for monthly activities (Table 1). Of all specimens caught, 3250 (67.8%) male and 1542 (32.2%) female were identified. Furthermore, 1108 (23.1%) from internal sites and 3684 (76.9%) were captured from external sites.

Twelve phlebotomine species were morphologically identified as sand flies fauna (6 *Phlebotomus*, 6 *Sergentomyia*) involving *P. papatasi*, *P. sergenti* sl, *P. caucasicus*, *P. alexandri*, *P. mongolensis*, *P. bergeroti*, *S. theodori*, *S. dentata*, *S. antennata*, *S. clydei*, *S. baghdadis*, and *S. sintoni*. The most abundant of these sand flies was *P. papatasi*, which was thus considered the dominant species too. *Sergentomyia baghdadis* and *P. sergenti* sl ranked second and third dominant after *P. papatasi* in this area. *Phlebotomus papatasi* was also the dominant species both indoor (86.7%) and outdoor (80.8%), and *S. baghdadis* (5.8%) was the dominant species in

outdoor-based observations of monthly activity study. The activity of these insects started in May and ended in September (Fig. 2), and the peaks activity were twice a year (mid-June and mid-August).

The kinetoplast DNA of the parasite *L. major* was detected in 26 (17.3%) out of the 150 female phlebotomine sand flies with nested PCR (Table 2). Twenty-five (19.2%) out of the 130 *P. papatasi* sand flies were infected with *L. major* (Fig. 3 and 4).

**Table 1.** Fauna and relative abundance of sand flies collected in Fasa, Fars Province, southern Iran, 2011

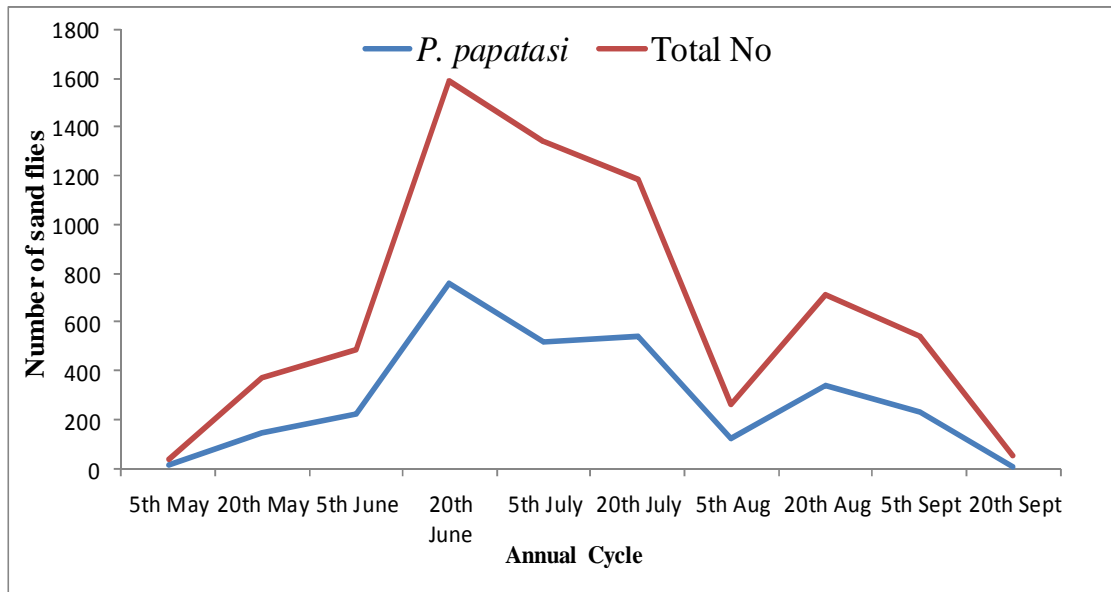
Sand fly species	Indoor n (%)	Outdoor n (%)	Male n (%)	Female n (%)	Total n (%)
<i>P. (Phlebotomus) papatasi</i>	267 (86.7)	652 (80.8)	687 (74.8)	232 (25.2)	919 (82.4)
<i>P. (Phlebotomus) bergeroti</i>	4 (1.3)	5 (0.61)	0.0	9 (100)	9 (0.8)
<i>P. (Paraphlebotomus) alexandri</i>	9 (2.9)	19 (2.35)	17 (60.7)	11 (39.3)	28 (2.5)
<i>P. (Paraphlebotomus) sergenti sl</i>	8 (2.6)	25 (3.09)	22 (66.7)	11 (33.3)	33 (2.95)
<i>P. (Paraphlebotomus) caucasicus</i>	3 (0.97)	11 (78.6)	14 (100)	0.0	14 (1.25)
<i>P. (Paraphlebotomus) mongolensis</i>	1 (0.3)	0.0	1 (100)	0.0	1 (0.1)
<i>S. (Sergentomyia) theodori</i>	5 (1.6)	21 (2.7)	5 (19.2)	21 (80.8)	26 (2.3)
<i>S. (Sergentomyia) dentata</i>	3 (0.97)	8 (1)	3 (27.3)	8 (72.7)	11 (1)
<i>S. (Sergentomyia) antennata</i>	0.0	1 (0.1)	1 (100)	0.0	1 (0.1)
<i>S. (Sergentomyia) sintoni</i>	0.0	2 (0.2)	2 (100)	0.0	2 (0.2)
<i>S. (Parrotomyia) baghdadis</i>	7 (2.3)	47 (5.8)	21 (38.9)	33 (61.1)	54 (4.8)
<i>S. (Sintonius) clydei</i>	1 (0.32)	16 (2)	11 (64.7)	6 (35.3)	17 (1.5)
<b>Total</b>	<b>308 (27.62)</b>	<b>807(72.38)</b>	<b>779 (69.9)</b>	<b>336 (30.1)</b>	<b>1115 (100)</b>



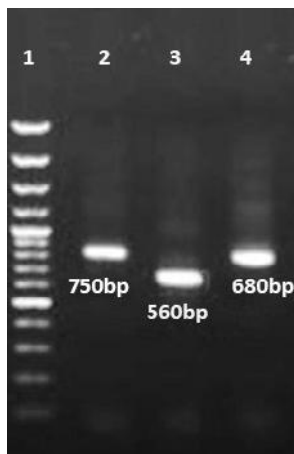
**Fig. 1.** Local map of the study area, Fasa, in Fars Province of southern Iran. The numbers 1–10 refer to sampling sites: 1-Amirhajiloo, 2-Sheshdeh, 3-Jelian, 4-Vaselabad, 5-Miandeh, 6-Fedeshkooyeh, 7-Sahrarood, 8-Kooshkeghazi, 9-Maghaberi, 10-Vakilabad

**Table 2.** PCR-based rate of infection among selected sand fly species in Fasa, Fars Province, southern Iran, 2011

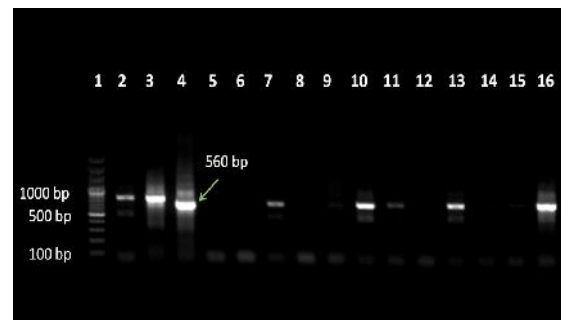
Sand fly species	Tested	Infected (%)
<i>P. (Phlebotomus) papatasi</i>	130	25 (19.2)
<i>P. (Paraphlebotomus) sergenti</i>	5	0 (0)
<i>S. (Sergentomyia) theodori</i>	10	1 (10)
<i>S. (Parrotomyia) baghdadis</i>	5	0 (0)
<b>Total</b>	<b>150</b>	<b>26 (17.3)</b>



**Fig. 2.** Seasonal activity of sand flies collected in Fasa, southern Iran, during 2011



**Fig. 3.** Nested PCR gel electrophoresis of reference strains with four distinct primers (CSBIXR, CSB2XF, LiR and 13Z) in 1.5% agar gel stained with ethidium bromide. Lane 1: 100 bp marker, Lane 2: *Leishmania tropica*, Lane 3: *L. major* and Lane 4: *L. infantum* standards



**Fig. 4.** Electrophoresis results produced by nested PCR of female sand fly specimens with four primers (CSBIXR, CSB2XF, LiR and 13Z) in 1.5% agarose gel stained with ethidium bromide. Lane 1: 100 bp marker, Lane 2: *Leishmania tropica*, Lane 3: *L. infantum*, Lane 5: negative control (male sand fly), Lanes 6, 8, 9, 12, and 14: parasite-free female *Phlebotomus papatasi*, Lane 15: uninfected female sand fly *P. sergenti*, Lanes 4, 7, 10, 11, 13 and 16: sand fly *P. papatasi* infected with the parasite *L. major*

## Discussion

Control of leishmaniasis necessitates studies on the epidemiology and ecology of this disease vectors and reservoir hosts. The entomological survey and epidemiological study are two important components of control against the spread of infectious agents. Common infection with *Leishmania* parasites in sand fly and human in the same place are important features for introducing the main vectors (Killick-Kendrick 1990).

Fasa district is one of the most endemic cutaneous leishmaniasis foci of Fars Province. In spite of control program, some 3224 people were affected by this disease during the period of 2006–2013 (Khosravani et al. 2016) and the disease foci still exist in this district. This study was the first on vectors of leishmaniasis in this area. The present study found 12 species of sand flies (6 *Phlebotomus* and 6 *Sergentomyia*). Based on these results, *P. papatasi* was the most abundant both indoors and outdoors. *P. papatasi* was introduced as the dominant species in this province (Salehi 1997, Kalantari 2003, Azizi et al. 2008, 2010, Davami et al. 2010). *Phlebotomus alexandri* was one of the identified species in this study, previously reported to be a visceral leishmaniasis vector in some parts of Iran (Azizi et al. 2008, Bakhshi et al. 2013). *Phlebotomus sergenti* sl was also identified in this study which is effective in the transmission of cutaneous leishmaniasis in Iran (Moin-Vaziri et al. 2007, Oshaghi et al. 2010). Sand fly activity from mid-May to the beginning of November had two peaks in late June and September and *P. papatasi* was the dominant species which was modulated with the activity design of other sand flies in this area and was almost consistent with the activity of sand flies in ZCL foci in Iran (Yaghoobi-Ershadi et al. 2001, Abai et al. 2007).

Molecular (PCR) method was used to detect *Leishmania* infection in sand flies. Some

150 unfed parous females were examined by nested PCR techniques and proved their infection to be *L. major* in 19.2% of *P. papatasi* species. Previous reports had proved *L. major* infection in *P. papatasi* in Iran (Rassi et al. 2011). This method was used since it was very easy compared to other methods if it was set up in the laboratory, large number of species can be examined in a short time. This method could not distinguish between amastigotes (those parasites without flagella in human) and promastigote (flagellated form of parasite in sand fly body) (Azizi et al. 2012b). In order to ensure transmission of parasite by sand flies, it is essential to examine parous or empty stomach female sand fly (Molyneux and Ashford 1983).

A single infection of *S. theodori* was found in this study. Natural promastigote infection of other species of *Sergentomyia* like *S. dentata* sand flies have previously been reported from Ardebil Province, northwest of Iran (Rassi et al. 1997), though this finding does not represent an evidence of this sand fly being a vector.

Evaluation of infection in sand flies was tested by PCR. This method has as high sensitivity and specificity as ELISA method used before (Maleki-Ravasan et al. 2009). The sand flies that were used in this study had mostly digested blood.

According to our studies, a consistent pattern of abundance and fauna in this region occurs with other ZCL foci in Iran. *Phlebotomus papatasi* is the main cutaneous leishmaniasis vector in this district. Epidemiologic study on reservoirs and human infection are essential for providing proper control program. The personal protection including the use of long-lasting insecticidal nets (LLIN), installing screens on windows and doors, using repellents, and environmental improvements such as waste fertilizers and trash as well as rodent control in houses are

the most important strategies in the leishmaniasis control program.

## Conclusion

*Phlebotomus papatasi* is considered as a main vector of zoonotic cutaneous leishmaniasis in Fasa, Fars Province, south of Iran.

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