

# *Phlebotomus papatasi* (Diptera: Psychodidae) as the Vector of *Leishmania Major* in Kharameh District, Southern Iran

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## Abstract

**Background:** Cutaneous Leishmaniasis (CL) is endemic in many parts of Iran. This study was conducted to investigate the fauna and some biologic factors of sand flies and detect CL vector(s) in Kharameh district which is one of the most important foci of the disease in Fars province, southern Iran.

**Methods:** To identify the fauna, a total of 1549 sand flies were collected from April 2014 to March 2015. To determine the monthly activity, sand flies were collected from indoor and outdoor areas of the lowland and the highland regions.

**Results:** Ten species of phlebotomine (four *Phlebotomus* spp. and six *Sergentomyia* spp.) were identified and *Phlebotomus papatasi* was the dominant species (53.45%). To determine the sand flies naturally infected by *Leishmania* spp., 188 female sand flies (145 *P. papatasi*, 29 *P. sergenti*, and 14 *P. alexandri*) were subjected to polymerase chain reaction (PCR) assay. Two (13.16%) specimens of *P. papatasi* were found to be positive for *Leishmania major*.

**Conclusions:** To the best of our knowledge, this is the first PCR detection of *L. major* within naturally infected *P. Papatasis* and fly as the main vector in this region of south Iran.

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**Keywords:** Sand flies, Fauna, Monthly activity, Kharameh, Iran

## Introduction

Leishmaniasis are endemic diseases in different parts of Iran. Cutaneous Leishmaniasis (CL) is an increasing public health problem in 15 provinces of the country.<sup>1</sup> CL was first described in southern Iran by Nadim and colleagues.<sup>2</sup> In this area, the yearly incidence of CL has increased over the last decade.<sup>2</sup> Sand flies are vectors of at least three kinds of diseases to human, the most important of which is leishmaniasis. Infection is transmitted to humans via biting of the infected female sand flies.<sup>3</sup>

*Leishmania major* is the main causative agent of wet lesions of CL. It is mainly found in the rural areas. In Iran, CL has also been found in the urban areas where the results of *Leishmania tropica* infection have been observed.<sup>4</sup> Both Anthroponotic (ACL) and

Zoonotic (ZCL) forms caused by *L. tropica* and *L. major* occur in Iran. It seems that ACL is restricted to some large and medium-sized cities whereas ZCL is endemic in rural areas of different parts of the country. Correct detection of the parasite(s) causing each case of CL is needed if the local measures for the control of the disease are to be effective.<sup>5</sup>

The characterizing techniques such as microscopic examination of direct smears and/or the culture of biopsies are not sensitive enough and diagnostic methods based on deoxyribonucleic acid (DNA) have now resulted in sensitive and quick detection of the micro organism possible.<sup>6</sup>

*Phlebotomus* species are the sole vector responsible for transmitting the disease in the old world. Characterizing of the vector in the endemic areas is vital for both chemotherapy and prognosis.<sup>7</sup>

Moreover, determination of *Leishmania* vectors and their biology is required for planning *Leishmania* control policies.<sup>8</sup> Sand flies frequencies and activities are influenced by temperature, relative humidity, soil moisture, and wind speed.<sup>9</sup> Temperature and moisture also affect the activities of sand flies.<sup>10</sup>

Fars province is one of the most important foci of leishmaniasis in Iran. In 2014, 510 cases of ZCL have been reported in Kharameh district (unpubl. obs.). In this article, the fauna, monthly activity, and geographic distribution of sand flies have been studied in this district.

## Materials and Methods

### Study Area

Fars province is located in southern Iran and has an area of 122 400 km<sup>2</sup>. The study was carried out in Kharameh district, located 80 kilometres northeast of Shiraz, the capital city of Fars province, southern Iran. This region is situated at 29° 30' 4" N, 53° 19' 15" E and 1500 m above the sea level.<sup>11</sup> Due to suitable biological conditions for *Leishmania* reservoir hosts, vectors and reservoirs diversity, Kharameh has always been considered as one of the CL endemic regions (Figure 1).

### Collection and Examination of Sand Flies

During April 2014 and March 2015, sand flies

were collected monthly by sticky paper traps (castor oil-coated white papers, 20×32 cm) from indoor and outdoor areas (rodent burrows, agricultural plantations surrounding the houses, etc.) in different areas of Kharameh district including villages of Ahmad-Abad and Korbal regions.

In each sampling, 30 sticky paper traps were set in the evening and collected in the next morning. Sand flies were mounted in Puri's medium.<sup>12</sup> Identification of the specimens was based on specific taxonomic criteria.<sup>13,14</sup> In determining the monthly activity of sand flies, they were caught from indoor and outdoor areas of the lowland and the highland locations during this period.

### DNA Extraction

Total genomic DNA of each female sand fly was extracted using the method described by Motazedian et al. (2002) with some modifications.<sup>15</sup> Each sample was homogenized in a 1.5 ml microtube containing 200 µl lysisbuffer [50 mM Tris-HCl (pH=7.6), 1 mM EDTA and 1% Tween 20] and 15 µl of proteinase K solution (20 µg/ml). The homogenate was incubated at 37°C overnight before 100 µl from a phenol: chloroform: isoamylalcohol mixture (25:24:1, by vol.) were added. It was centrifuged at 12,000 rpm for 5 min and the DNA in the supernatant was precipitated with 400 µl cold pure ethanol, re-suspended in 50 µl ddH<sub>2</sub>O and stored at -20°C before use in the PCR

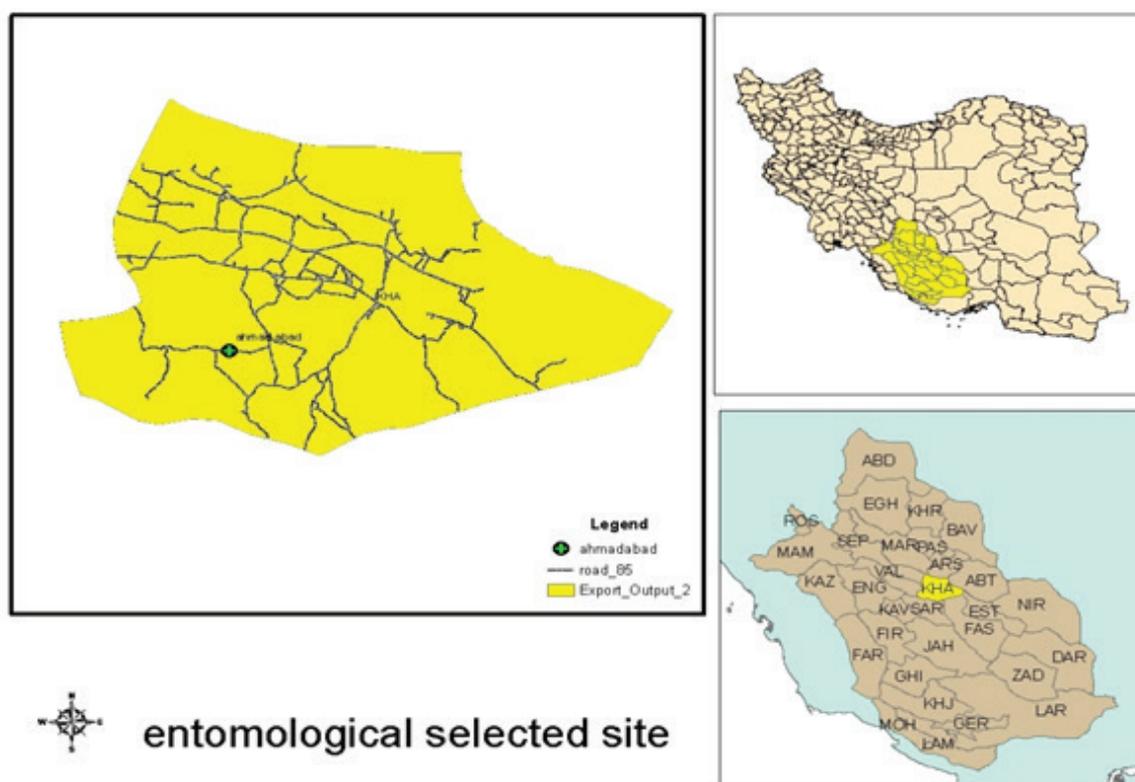


Figure 1: Map of Kharameh, Fars province, southern Iran

assay to detect *Leishmania*-specific kDNA. Only 5 µl portions of DNA extracts were processed for PCR.<sup>15</sup>

#### PCR Assay

*Leishmania* typing was carried out using the standard PCR procedure as described by Azizi et al. (2008). A set of two primers (LINR4 and LIN17) were also designed within the conserved area of the minicircle DNA of *Leishmania* parasite. This set of primers including LINR4 (5'-GGG GTT GGT GTA AAA TAG GG-3'), and LIN17 (5'-TTT GAA CGG GAT TTCTG-3') was used in a PCR technique with a slight modification.<sup>16</sup> Reference strains of *L. major* (MHOM/IR/54/LV39), and *L. tropica* (MHOM/IR/89/ARD2) were used as standards. PCR protocol was used to amplify the variable region of the minicircle DNA of any *Leishmania* in the sand fly midgut and involved a one-round PCR. The amplification reaction mixture contained 250 µM of each deoxynucleoside triphosphate (dNTP), 1.5 mM MgCl<sub>2</sub>, 1 unit Taq polymerase (Cinagene, Tehran), 1 µM primer LINR4, 1 µM primer LIN17 and 5 µl of DNA extract in 1X PCR buffer (Cinagene, Tehran, Iran), in a final volume of 25 µl. This reaction mixture was incubated in a CGI-96 thermocycler (Palm-cycler, CGI-96, Genetics Biotech Asia, India) set to run for 5 min at 94°C, followed by 30 cycles, each one consisting of 30 s at 94°C, 30 s at 55°C, 60 s at 72°C, and a final extension at 72°C for 10 min and kept at 4°C. Negative controls (DNA extracts from male sand flies and aliquots of ddH<sub>2</sub>O) were run in each PCR protocol to detect possible contamination which could cause false positive results.<sup>16</sup>

#### Agarose-Gel Electrophoresis

A 5 µl sample of each PCR end product was subjected 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-weight markers and the relevant second-round products for the *L. major* and *L. tropica* standards.

## Results

In this study, among 1549 sand flies collected, four species of *Phlebotomus* and six species of *Sergentomyia* were identified to comprise *P. papatasi*, *P. bergeroti*, *P. sergenti*, *P. alexandri*, *Sergentomyia antennata*, *S. clydei*, *S. Sintoni*, *S. theodori*, *S. baghdadis*, and *S. Squamipleuris* (Table 1).

Monthly activity of the dominant species (*P. papatasi*) and the total flies are shown in Table 2. To determine the sand flies naturally infected by *Leishmania* spp., 188 female sand flies consisting of 145 *P. papatasi*, 29 *P. sergenti*, and 14 *P. alexandri* were dissected and evaluated microscopically using Giemsa-stained slides. Natural infections of two out of 145 (1.38%) *P. papatasi* were positive for *Leishmania major* (Figure 2).

## Discussion

Cutaneous leishmaniasis is the main common vector-borne disease problem in Iran, where most cases known as zoonotic CL (ZCL) are caused by *L. major*.<sup>6,17</sup> Fars province is enriched in sand flies fauna because of its high bio-ecological diversity. *P. major*, *P. keshishiani*, *P. alexandri*, and *P. papatasi* have been described as the vector(s) of visceral leishmaniasis and ZCL in this province, respectively.<sup>6,7,18</sup> *P. papatasi* has been described as the main and proven vector of *L. major* in all endemic foci of this province.<sup>3,19</sup> In this study, *P. papatasi* was the main species (53.45%). Moreover, the high activity levels of sand flies were observed during June and September.

Due to high bio-ecological diversity of sand flies, many species of *Phlebotomus* such as *P. papatasi*, *P. major*, *P. keshishiani*, and *P. alexandri* are reported as the vector(s) of CL and VL in this focus.<sup>7,18</sup>

Detection of *Leishmania* vectors and their biology is necessary for planning *Leishmania* control strategies (Killick-Kendrick, 1999). Molecular-based methods using PCR are well-organized and reliable

**Table 1:** The types and numbers of sand flies collected in each of the ten study regions in Kharameh district, southern Iran

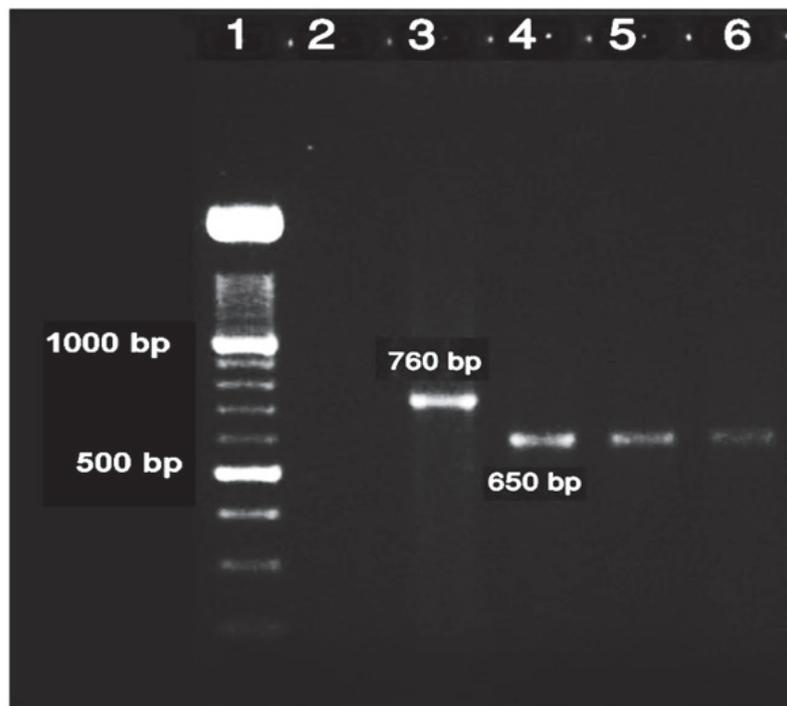
Genus and species	No & % of sandflies collected in Kharameh district						Total (No.) (%)	
	Male			Female			♂♀	
	Outdoors	Indoors	Total	Outdoors	Indoors	Total		
<i>P.<sup>1</sup>(P.) papatasi</i>	157 (10.14)	380 (24.53)	537 (39.06)	100 (6.46)	191 (12.33)	291 (18.79)	828	53.45
<i>P. (P.) bergeroti</i>	42 (2.71)	43 (2.78)	85 (5.49)	44 (2.76)	35 (2.26)	79 (5.10)	164	10.59
<i>P. (Para.)<sup>2</sup>sergenti</i>	38 (2.45)	24 (1.55)	62 (4)	21 (1.36)	16 (1.03)	37 (2.38)	99	6.39
<i>P. (Para.) alexandri</i>	12 (0.77)	9 (0.58)	21 (1.36)	5 (0.32)	3 (0.19)	8 (0.52)	29	1.87
<i>S.<sup>3</sup> (S.) antennata</i>	85 (5.49)	117 (7.55)	202 (13.04)	66 (4.26)	52 (3.36)	118 (7.61)	320	20.66
<i>S. (Sin.)<sup>4</sup>clydei</i>	11 (0.71)	8 (0.52)	19 (1.23)	4 (0.26)	2 (0.12)	6 (0.39)	25	1.61
<i>S. (S.) sintoni</i>	7 (0.45)	9 (0.58)	16 (1.03)	4 (0.26)	4 (0.26)	8 (0.52)	24	1.55
<i>S. (S.) theodori</i>	9 (0.58)	7 (0.45)	16 (1.03)	3 (0.19)	2 (0.12)	5 (0.32)	21	1.36
<i>S. (Parr.)<sup>5</sup>baghdadis</i>	9 (0.58)	5 (0.32)	14 (0.90)	2 (0.12)	4 (0.26)	6 (0.39)	20	1.29
<i>S. (Gr.)<sup>6</sup>squamipleuris</i>	4 (0.26)	3 (0.19)	7 (0.45)	7 (0.45)	5 (0.32)	12 (0.77)	19	1.23
Total	374 (38.2)	605 (61.8)	979 (63.20)	256 (44.9)	314 (55.1)	570 (36.80)	1549	100

♂ Female; ♀ Male; <sup>1</sup>*Phlebotomus*, <sup>2</sup>*Paraphlebotomus*, <sup>3</sup>*Sergentomyia*, <sup>4</sup>*Sintonius*, <sup>5</sup>*Parrotomyia*, <sup>6</sup>*Grassomyia*

**Table 2:** Monthly activity of sand flies caught in Kharameh district, Fars province, southern Iran, 2014

Months	<i>P. papatasi</i>	<i>P. bergeroti</i>	<i>P. sergenti</i>	<i>P. alexandri</i>	<i>S. antennata</i>	<i>S. clydei</i>	<i>S. sintoni</i>	<i>S. theodori</i>	<i>S. baghdadis</i>	<i>S. squamipleuris</i>
April	10	0	0	0	2	3	0	0	0	0
May	130	24	8	0	38	5	0	0	0	0
June	244	44	35	13	104	10	0	6	0	3
July	156	36	13	2	60	2	7	4	0	0
August	112	20	10	3	48	3	4	0	5	7
September	160	37	29	11	68	6	13	11 (0.71)	15	9
October	16	3	0	0	0	0	0	0	0	0
November	0	0	0	0	0	0	0	0	0	0
December	0	0	0	0	0	0	0	0	0	0
January	0	0	0	0	0	0	0	0	0	0
February	0	0	0	0	0	0	0	0	0	0
March	0	0	0	0	0	0	0	0	0	0
Total	828 (53.45)	164 (1.59)	95 (6.13)	29 (1.87)	320 (20.66)	29 (1.87)	24 (1.55)	21 (1.36)	20 (1.29)	19 (1.23)

No. & % of sand flies



**Figure 2:** Gel electrophoresis PCR results of *Leishmania* parasites isolated from sand flies in Kharameh district using the primers Lin17 and Lin 4; marker (100-bp ladder) (lane 1), a male sandfly as negative control (lane 2), reference strain of *Leishmaniatropica* (760 bp) (lane 3), reference strain of *L. major* (650 bp) (lane 4), *L. major* isolated from *Phlebotomuspapatasi* in Ahmad-Abad in Kharameh district (lanes 5, and6).

tools for the detection of *Leishmania* spp. They are more sensitive and specific than microscopic methods (Aransay et al., 2000). Although molecular methods such as PCR methods and sequencing have several advantages and drawbacks, when they are associated with morphological characterization of the sand flies, they will be a powerful epidemiological tool in determination of the number of sand flies infected with *Leishmania* spp. in nature (Aransay et al., 2000). On the basis of our findings, this is the first molecular detection of *L. major* within naturally infected *P. papatasias* the main vector of ZCL in this region of Fars province, southern Iran.

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**Conflict of Interest:** None declared.

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