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ORIGINAL ARTICLE

Study of fauna, activity patterns and *Leishmania* infection rate of phlebotomine sand flies in Western Iran

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Abstract Cutaneous leishmaniasis is a crucial vector-borne disease caused by various species of *Leishmania* and is transmitted by several species of sandflies. The present study was conducted to describe sand fly fauna on vectors of leishmaniasis and performing molecular identification of *Leishmania* isolates from them on the Iran–Iraq border. Entomological surveys were done from May to October 2016–2018 in 2 counties (Mehran and Dehloran) of Ilam province, west of Iran. Sandflies were collected by 40 Sticky Traps at each station. Samples were mounted for species identification using morphological characters of the head and abdominal terminalia. DNA was extracted from *Phlebotomus papatasi* females, and *Leishmania* isolates were identified through PCR on minicircle kDNA, followed by sequencing. A total of 5592 sandflies including 2 genera of *Phlebotomus* and *Sergentomyia* comprising 8 species of sand flies were detected. *Leishmania major* infection was detected in 3.33% of 300 tested female sandflies. *Phlebotomus papatasi* was predominant in outdoor and indoor resting places. *Phlebotomus papatasi* was determined as dominant vector of *Leishmania major* infection in Mehran and Dehloran counties, West of Iran. It seems the composition of sandfly species in the study area is almost similar to the other parts of Iran. A detailed description of the epidemiology and ecology of Phlebotomine sand flies needs to be established to accomplish effective vector control programs.

Keywords *Leishmania* · Psychodidae · *Phlebotomine* · Sand fly · Iran

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Abbreviations

CL	Cutaneous leishmaniasis
ST	Sticky Traps
ZCL	Zoonotic cutaneous leishmaniasis
GIS	Geographical Information System
SDS	Sodium Dodecyl Sulfate
PCR	Polymerase chain reaction

Introduction

Phlebotomine sandflies (Diptera: Psychodidae) are proven vectors responsible for the transmission of different species of *Leishmania*, the etiologic agents of leishmaniasis (Moncaz et al. 2012; Chagas et al. 2018), in many geographical regions of the world including the countries of

Middle East (Salam et al. 2014). In this area, Iran is one of the most important countries in terms of cutaneous leishmaniasis cases, so the disease is endemic in 17 out of 31 provinces, and more than 20,000 new cases are reported annually from different parts of the country (Yaghoobi-Ershadi 2012; Arjmand et al. 2014; Salehi et al. 2014; Hashemi et al. 2016). Zoonotic cutaneous leishmaniasis (ZCL) is a critical health problem with 80% of cases reported in Iran (Yaghoobi-Ershadi 2012). A very important aspect of the control of leishmaniasis is knowledge of wild cycles of *Leishmania*. Previous studies conducted in Iran have demonstrated that *Phlebotomus papatasi* (*P. papatasi*) is the main vector of *Leishmania major* as a causative agent of ZCL (Yavar et al. 2011; Rafizadeh et al. 2016; Zivdari et al. 2018). However, other species such as *P. caucasicus*, *P. bergeroti* (Parvizi et al. 2013; Azizi et al. 2016a), *P. mongoliensis* (Akhoundi et al. 2013) and *P. alexanderi* (Bakhshi et al. 2013) are considered as secondary vectors. Ilam province is located in the west of the country and has been concerned as one of ZCL foci. Residents of Mehran and Dehloran counties and their suburbs, as parts of Ilam province are at the risk of leishmaniasis and in recent years, the incidence of the disease has been increased. Annual passage of pilgrims from Western borderline of Iran and Iraq, especially Mehran and Dehloran counties and presence of enzootic ZCL foci in this area has increased the risk of developing CL tremendously (Saberi et al. 2018).

Several environmental factors may influence, directly or indirectly, the distribution of sand flies (Rispail et al. 2002; Guernaoui et al. 2006). With increasing accessibility to new tools and techniques, it has become possible to improve remedial measures, for example, the use of Geographical Information System (GIS) techniques can provide useful information in leishmaniasis control programs and evaluating human disease risk (Kahime et al. 2015; Ostad et al. 2016). To the best of our knowledge, despite the extensive and diverse sand fly distribution range in geographical borders of Ilam, there is no precise report on environmental variables related to the population structure of sand flies. This study was conducted to determine fauna and annual activity patterns of sandflies considering environmental and climatic fluctuations as well as detecting and identifying the *Leishmania* parasite by a semi-nested PCR method in ZCL focused on the border of Iran and Iraq, during 2016–2018.

Methods

Ethics approval

All applicable international and institutional guidelines for conducting the study in 'the houses and domestic animal shelters' were followed. The study protocol was approved by the ethical committee of the Isfahan University of Medical Sciences, (IR.MUI.MED.REC.1398.523). The guideline of the Institutional Animal and Ethics Committee, Isfahan University of Medical Sciences was used to work in this study.

Study area

Entomological surveys were conducted on a large geographical scale in Ilam Province, located west of Iran. This area, bordering Iraq, lies within latitudes of 31°58′ and 34°15′ N and longitudes of 45°24′ and 48°10′ E (Fig. 1). The studied area has a warm and dry climate with hot summers and mild, short winters. The average annual rainfall is 181.3 mm and the absolute temperature varied from − 3 to 56 °C. Mehran and Dehloran counties were considered as main sampling areas, however, districts and villages of these two cities including Konjan cham, Changuleh, Chalab, Musiyan, Chamhendi, and Dasht-e Abbas were also monitored.

Sandfly collection

Sandflies were collected from 44 different zones in their active season, from May to October 2016–2018. To do this, 40 Sticky Traps (ST) consisting of sheets soaked with castor oil were used for indoor (inside the houses and domestic animal shelters), outdoor (outside the settlements) resting places, and near entries to rodents burrows. The average temperature and relative humidity of each sampling month were obtained from the nearest meteorological station located in Mehran and Dehloran counties. Trapped sand flies were detached from paper sheets using fine-pointed needles, and were rinsed in acetone to remove remaining castor oil, and then were preserved in 70% ethanol until processing.

Species identification

After washing in a 1% detergent solution for 5 min, all sand flies were dissected under a loop microscope (Aransay et al. 2000). For all samples, the head and posterior section of the abdomen were mounted in the Puri's clearing medium for morphological identification based on standard keys (Seyedi-Rashti and Nadim 1967; Lewis 1982; Seyedi-Rashti et al. 1992), and remaining body segments of female sand flies including blood-feds were used for *Leishmania* detection by using the molecular method.

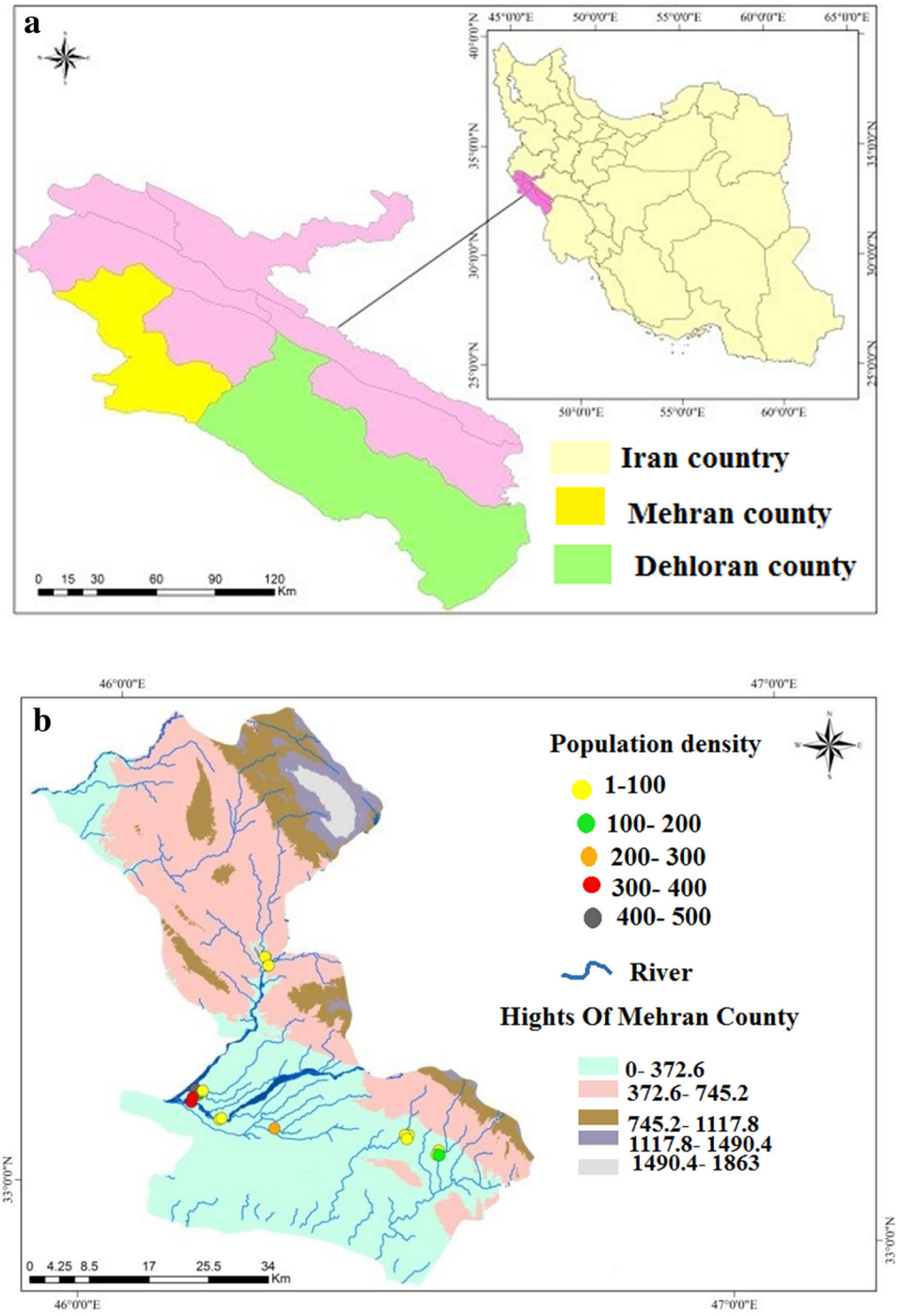


Fig. 1 Map of the area of study and sandflies population density at the sampling location

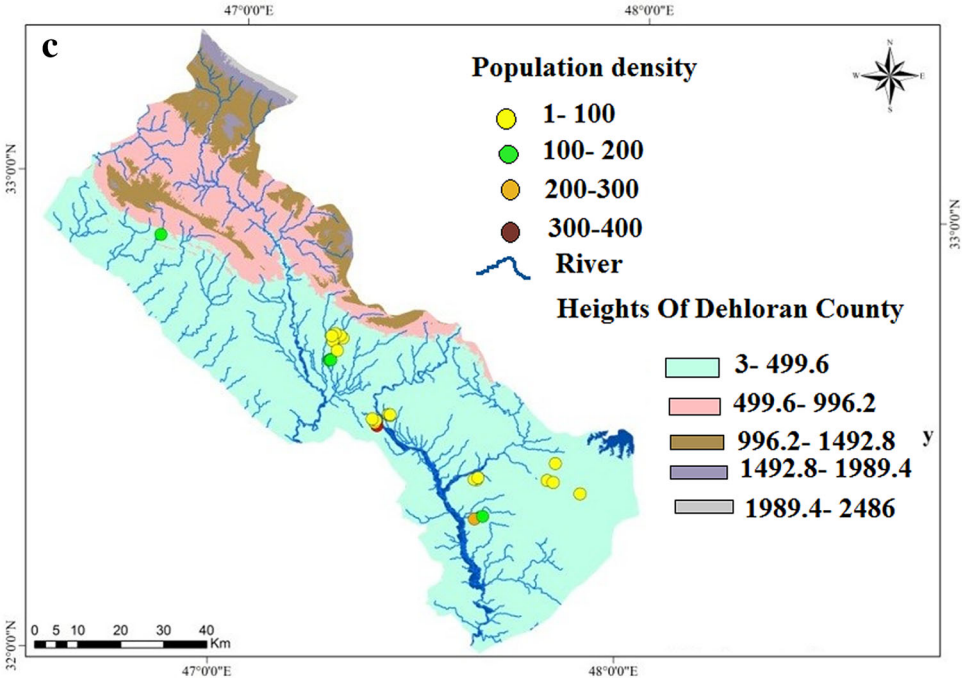
GIS database and data analysis

The effect of environmental variables on the habitat of *P. papatasi* as the main vector of leishmaniasis in studied areas was investigated using GIS analysis (ArcGis v.10 software) based on their influence on leishmaniasis ecology and vectors.

Molecular survey

Molecular procedures were applied to detect *Leishmania* parasites in 300 *P. papatasi* species (100 female sand flies per year).

Fig. 1 continued



DNA extraction

DNA was extracted from collected samples using a modified classical phenol–chloroform extraction method applied in the study by Casaril, et al. It is noteworthy that, the proposed protocol has been confirmed to have a significantly better performance than commercial kits (Casaril et al. 2017).

The protocol was as follows: Samples were homogenized completely in 10 µl of 20% Sodium Dodecyl Sulfate (SDS), 200 µl of Tris–EDTA (TE), a few pieces of plastic pestles was used for grinding of the specimen. Then, 5 µl of proteinase K (20 mg/ml) was added to homogenized samples. The tubes were incubated in a dry bath at 42 °C for 24 h. 200 µl of phenol:chloroform: isoamyl alcohol was added and the samples were centrifuged at 12,000 rpm for 5 min. The supernatant containing DNA was collected and 20 µl of 3 M sodium acetate and 400 µl of ice-cold 100% ethanol was added, and the tubes were incubated overnight at − 20 °C. Then, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the sediments were washed three times with 500 µl of 70% ethanol, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. Following ethanol evaporation at ambient temperature, the samples were re-suspended in 50 µl of 1 × TE and were stored at − 20 °C until use.

Semi-nested PCR technique

A set of primers including LINR4 (forward) (5′-GGG GTT GGT GTA AAA TAG GG - 3′), LIN17 (reverse) (5′-TTT GAA CGG GAT TTC TG- 3′), and LIN19 (reverse) (5′-CAG AAC GCC CCT ACC CG- 3′) were used in a semi-nested PCR technique to amplify the conserved area of minicircle kDNA of *Leishmania* isolates (Aransay et al. 2000).

The used thermocycler (Perkin Elmer (PE) GeneAmp®PCR) was set for 5 min at 94 °C followed by 17 cycles, each consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C, which continued with a final extension lasting for a further 10 min. Cross-contamination was monitored by DNAs extracted from male sand flies and reference strains of *L. major* (MHOM/IR/54/LV39) were used as positive controls.

Results

Species composition and activity patterns of sand fly

A total of 5592 sand flies were collected (among which 3322 species were trapped in Mehran and 2270 in Dehloran Counties) during the 3 survey periods: 2016 (n = 1784; 31.9%); 2017 (n = 2345; 41.9%); and 2018 (n = 1463; 26.2%). All sand flies were captured by sticky traps, and there were no statistically significant differences among the 3periods (Kruskal–Wallis H-test: $\chi^2 = 0.23$, $df = 2$,

Table 1 Geographical distribution of different sand fly species in Mehran and Dehloran Counties, Ilam province, Iran, 2016–2018

Year	*P. papatasi*			*P. alexandri*			*S. squamipleuris*			*S. iranica*			*S. dentata*			*S. antennata*			*S. theodori*			*S. clydei*		
	3*	2*	1*	3	2	1	3	2	1	3	2	1	3	2	1	3	2	1	3	2	1	3	2	1
Konjan Cham	128	189	98	27	34	22	1	0	0	0	2	0	9	5	0	0	0	0	0	0	0	0	0	0
Total (%)	415 (8.89)			83 (16.5)			1 (1.81)			2 (2.66)			14 (5.42)			0			0			0		
Mehran*	719	891	504	59	75	41	20	4	4	10	8	16	38	33	28	4	6	4	0	0	0	0	0	0
Total (%)	2114 (45.4)			175 (34.9)			28 (50.9)			34 (45.36)			99 (38.37)			14 (60.9)			0			0		
Changuleh	108	93	77	12	27	7	0	1	0	2	0	1	3	9	2	0	0	1	0	0	0	0	0	0
Total (%)	278 (5.95)			46 (9.18)			1 (1.81)			3 (4)			14 (5.42)			1 (4.34)			0			0		
Dehloran*	123	240	167	19	41	12	0	6	3	0	4	6	8	27	9	0	3	0	0	2	0	0	0	1
Total (%)	530 (11.45)			72 (14.34)			9 (16.46)			10 (13.33)			44 (17.05)			3 (13.03)			2 (25)			1 (20)		
Musiyan	225	202	138	17	38	9	4	3	2	4	7	9	21	22	19	0	3	2	0	6	0	0	3	1
Total (%)	565 (12.2)			64 (12.74)			9 (16.46)			20 (26.66)			62 (24.03)			5 (21.73)			6 (75)			4 (80)		
Chamhendi	93	187	126	10	15	9	6	1	0	0	0	4	6	5	5	0	0	0	0	0	0	0	0	0
Total (%)	406 (8.8)			34 (6.77)			7 (12.92)			4 (5.33)			16 (6.2)			0			0			0		
Dasht-e Abbas	99	138	121	5	12	11	0	0	0	0	1	1	4	2	3	0	0	0	0	0	0	0	0	0
Total (%)	358 (7.67)			28 (5.57)			0			2 (2.66)			9 (3.48)			0			0			0		

*3:2018, 2: 2017, 1: 2016

Table 2 Frequency of sandflies species according to places of trapping in Mehran and Dehloran Counties, Ilam province, Iran, 2016–2018

Genus/species	Indoor (%)	Outdoor (%)	Rodent burrow (%)	Total (%)
P. papatasi	1539 (82.1)	2191 (80.9)	936 (93)	4666 (83.44)
P. alexandri	273 (14.5)	169 (6.23)	60 (6)	502 (8.97)
S. squamipleuris	12 (1.25)	41 (1.5)	2 (0.19)	55 (0.98)
S. iranica	14 (0.5)	58 (2.1)	3 (0.29)	75 (1.34)
S. dentate	32 (1.5)	222 (8.18)	4 (0.36)	258 (4.62)
S. antennata	3 (0.1)	18 (0.66)	2 (0.19)	23 (0.42)
S. theodori	–	8 (0.29)	–	8 (0.15)
S. clydei	1 (0.05)	4 (0.14)	–	5 (0.08)
Total	1874 (100)	2711 (100)	1007 (100)	5592 (100)

$P = 0.88$). Among total trapped flies, 5168 (92.4%) of them belonged to the *Phlebotomus* genus and 424 (7.6%) of them belonged to *Sergentomyia*. Concerning the 2 studied regions, the highest number of sand flies was found in the central region of Mehran County (n = 2114), followed by the Musiyan region in Dehloran County (n = 565) (Table 1).

Only 2 genera of *Phlebotomus* and *Sergentomyia* comprising 8 species were identified in this region, which were as follows: *P. papatasi* (n = 4666; 83.44%), *P. alexandri* (n = 502; 8.97%), *S. squamipleuris* (n = 45; 0.98%), *S. iranica* (n = 75; 1.34%), *S. dentata* (n = 258; 4.62%), *S. antennata* (n = 23; 0.42%), *S. theodori* (n = 8; 0.15%), and *S. clydei* (n = 5; 0.08%) (Table 2).

Phlebotomus papatasi was found as predominant captured species (n = 4666; 83.44%), which was consistent with other previous studies conducted in this area and other parts of the country [25, 26], and the highest number of this species was trapped during 2018. Also, *P. papatasi* was dominant species in indoor (82.1%), outdoor places (80.9%), and rodents burrows (93%) (Table 2).

Activity patterns of *P. papatasi,* as the main vector of ZCL in Iran, were evaluated concerning environmental factors (Table 3). *P. papatasi* population size started to increase in May and reached its peak in September, and then it decreased sharply in October during the 3 survey periods (Fig. 2).

As shown in Fig. 1, phlebotomines were collected at altitudes between 81 - 373 m, and statistically significant

Table 3 Statistical relationship between *P. papatasi* population size with some climatic variables in Mehran and Dehloran Counties, Ilam province, Iran, 2016–2018., during the period 2016–2018

Variables	2016		2017		2018	
	P	R	P	R	P	R
Min temp	0.001	0.823	0.003	0.697	0.005	0.661
Max temp	0.001	0.823	0.001	0.771	0.005	0.661
Mean temp	0.001	0.823	0.003	0.697	0.005	0.661
Min RH	0.003	−0.698	0.002	−0.736	0.019	−0.550
Max RH	0.001	−0.823	0.005	−0.661	0.033	−0.510
Mean RH	0.001	−0.811	0.006	−0.648	0.028	−0.523
Monthly rainfall	0.02	−0.592	0.005	−0.690	0.822	0.086
Wind speed	0.3	0.389	0.53	−0.234	0.559	−0.215

There was not significant correlation in values underlined

R = Correlation coefficient

RH = Relative humidity

Temp: temperature

differences were observed between the abundance of *P. papatasi* and the altitudes under study (Kruskal–Wallis H-test: $\chi^2 = 13.15$, $df = 3$, $P = 0.004$). Figure 1 presents the geographical distribution of the *P. papatas* population according to altitude and some environmental variables.

Leishmania-infected *P. papatasi* and parasite typing by kDNA semi-nested PCR

The rate of *Leishmania* natural infection in 300 *P. papatasi* females was evaluated by semi-nested PCR using mini-circle kDNA, and this fragment was amplified in 4 out of 150 (2.66%) female sand flies that did not contain a blood meal when collected, and in 6 out of 150 (4%) samples that contained blood (Table 4).

The size of all amplified products was about 650 bp on 1.5% agarose gel, and all cases were detected as *L. major* (Fig. 3). To confirm the identification of samples, Two PCR products were sequenced and subjected to NCBI-BLAST analysis for homology (http://blast.ncbi.nlm.nih.gov). The sequences showed 100% identity to the published strain under the accession number of AB678349.1.

Discussion

The current study was conducted to assess species composition and activity patterns for season and landscapes of sand flies in 2 endemic regions of cutaneous leishmaniasis, in western Iran. These areas are parts of the strategic borders of Iran and Iraq countries, and annual thousands of passengers from both countries travels to these areas for religious and commercial purposes. Infection of these peoples can be the source of leishmaniasis spread after coming back toward their localities.

Phlebotomus papatasi (83.44%) was the predominant species and *P. alexandri* (8.97%) was the second dominant species in terms of abundance, which was similar to the results of a study conducted elsewhere in the regions near to the studied area (Ebrahimi et al. 2016).

The presence of *P. alexandri* was not reported in 2 studies carried out by Moradi and Rassi (2018) and Vahabi et al. (Vahabi et al. 2016) in some areas also covered by the present study. *P. papatasi* was the most frequent species in both indoor and outdoor resting places such as rodents burrows indicating that this species plays a significant role as the main vector in this region (Yaghoobi-Ershadi 2012; Azizi et al. 2016b). There were changes in composition and activity patterns of sand flies during the 3 survey periods, probably attributing to the fact that ecological variables of the studied area are not uniform. The spread of leishmaniasis in many areas can be associated with ecological factors and climatic changes that favor the increase in vector population (Aspock et al. 2008).

Geographical Information System (GIS)-based analysis was used to assess the relationships between environmental variables and the distribution of *P. papatasi*. This species has been incriminated as the main vector of *L. major* (causative agent of ZCL) in various districts of Iran (Parvizi and Ready 2008; Afshar et al. 2011).

Climate factors (rainfall, winds, temperature, and relative humidity) can influence the creation of resting and breeding sites for sand fly species (Belen and Alten 2006; Kassem et al. 2012). The seasonal dynamics of *P. papatasi* showed a similar abundance and activity peak in

Table 4 Frequency of natural *Leishmania* infected *P. papatasi* based on abdominal position and collected areas variables in Mehran and Dehloran Counties, Ilam province, Iran, 2016–2018

Location	Abdomen position	Number	Cases positive (%)
Indoor	Contained blood	50	1 (2)
	No blood	50	1 (2)
Outdoor	Contained blood	50	2 (4)
	No blood	50	1 (2)
Rodent burrow	Contained blood	50	3 (6)
	No blood	50	2 (4)
Total		300	10 (3.33)

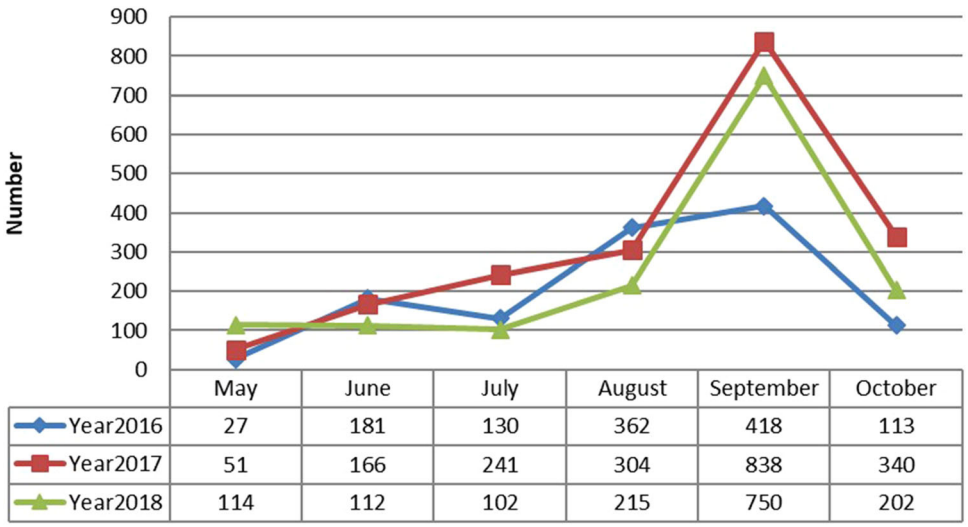


Fig. 2 Monthly changes of *P. papatasi* population from 2016 to 2018 in Mehran and Dehloran Counties, Ilam province, Iran

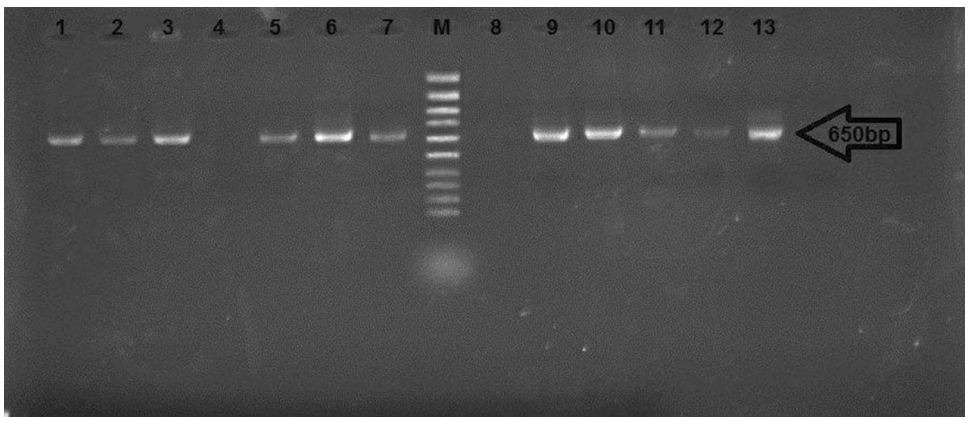


Fig. 3 Agarose gel electrophoresis of *Leishmania* isolates from sandflies in semi-nested PCR using the primers LINR4/LIN17and LIN19: Lanes 1–3, 5–7,9 and 11–13 *L. major* isolates obtained from sandflies; Lane M DNA size marker; Lane 4 and 8 negative control; Lane 10 *L. major* (positive control)

September (Fig. 2). Accordingly, people are required to take care of themselves during this period to prevent sandfly bites. There was no significant difference between mean temperatures in 3 survey periods. However, a maximum peak of captures was found in September 2017 (Fig. 2), the month with the lowest mean RH. Statistical analysis indicated a positive correlation between average monthly rainfalls, minimum, maximum, and mean monthly temperature and abundance of *P. papatasi* in 2016 and 2017 (Table 3). There was no such correlation for monthly rainfall in 2018, mostly attributing to environmental management actions such as spraying and drying up wetlands, conducted by regional health centers for the prevention of CL prevalence during 2018. The correlation between RH and temperature has been previously reported in

entomological studies carried out in other parts of the country (Mollalo et al. 2018).

Other studies performed in different parts of the world showed that altitude influences the spatial distribution and density of the *P. papatasi* (Guernaoui et al. 2005). In the present study, the abundance of *P. papatasi* was negatively correlated with the increase in altitude, although this species was distributed in sampling location at different altitudes. Belen, et al. also reported that this species can be found at altitudes ranging from near sea level to higher than 1100 m (Belen and Alten 2006). Despite the increasing annual prevalence of leishmaniasis in western Iran, there is insufficient evidence regarding the identification of species vectors. This study was conducted to elucidate the epidemiological aspects of sand flies and identify the *Leishmania* species in *P. papatasi* in border areas of the country. Finding the vectors infected with parasites is a basic way to control leishmaniasis.

The infection rate varies between the foci, and it is dependent on the techniques used for *Leishmania* detection. Parvizi, et al. compared nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kDNA for detection of *L. major* in peridomestic *P. papatasi* in Isfahan province (Iran). They found that the second method was more sensitive for the detection of *L. major* in wild-caught sandflies (Parvizi et al. 2005).

Herein, natural infection of *P. papatasi* was reported using semi-nested PCR of minicircle kDNA, and 10 isolates (3.33%) were identified as positive *Leishmania* parasites. 50% of infected samples were trapped near rodents burrows, which could be due to the high infection rate of rodents in the studied area. Comparison of sequences in PCR results with other sequences submitted in Worlds Gene Bank also confirmed that all cases were only infected with *L. major*, which was in line with another study conducted on another region of the country (Parvizi et al. 2012). In a similar study carried out in the northeast of Iran, in addition to *P. papatasi*, *Leishmania* infection was confirmed in *P. caucasicus* and *P. mongolensis* [38]. Contrary to some studies conducted in Iran, nonpathogenic *Leishmania* sp. such as *L. turanica* and *L. gerbilli* were not found in our study. The major role of these parasites in the survival of *L. major* in vectors and reservoirs has also been discussed (Sharbatkhori et al. 2014).

Conclusion

In our study, *P. papatasi was the predominant species* and it seems to be the main vector of leishmaniasis in studied area. This study is an updated report on phlebotomine sand fly distribution in western Iran according to some ecological factors results of which can be used to determine the areas at risk of leishmaniasis infection and developing efficient control strategies.

Participants

All applicable international and institutional guidelines for conducting the study in 'the houses and domestic animal shelters' were followed. The study protocol was approved by the ethical committee of the Isfahan University of Medical Sciences, (IR.MUI.MED.REC.1398.523). The guideline of the Institutional Animal and Ethics Committee, Isfahan University of Medical Sciences was used to work in this study.

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Author contributions All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this article.

Compliance with ethical standards

Conflict of interest All authors declare that there are no conflicts of interest.

Ethics approval and consent to participate All applicable international and institutional guidelines for conducting the study in 'the houses and domestic animal shelters' were followed. The study protocol was approved by the ethical committee of the Isfahan University of Medical Sciences, (IR.MUI.MED.REC.1398.523). The guideline of the Institutional Animal and Ethics Committee, Isfahan University of Medical Sciences was used to work in this study.

Consent for publication Not applicable.

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