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ORIGINAL ARTICLE

Bionomics of phlebotomine sand flies species (Diptera: Psychodidae) and their natural infection with *Leishmania* and *Crithidia* in Fars province, southern Iran

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Abstract Phlebotominae sand flies are involved in human diseases, such as leishmaniasis, and cause a considerable number of deaths every year. Besides, some of them have been identified as allergen sources or the potential mechanical vectors related to nosocomial infections. The present study aimed to assess the monthly activity, fauna, and detection of protozoan agents in phlebotomine sand flies using polymerase chain reaction (PCR) in re-emerging zoonotic cutaneous leishmaniasis foci of Shiraz and Kharameh in Fars province, southern Iran during 2016–2017. To determine the monthly activity, sand flies were caught from indoors and outdoors of both studied areas. Afterward, all female phlebotomine sand flies were processed for DNA extraction and PCR assays for *Leishmania* and *Crithidia* detections. During the study, 6975 sand flies of 16 species (eight *Phlebotomus* and eight *Sergentomyia* species) were caught in both foci. Sand flies' monthly activities started in early April and terminated in late November and October. Additionally, two active peaks of sand flies were observed in both foci; first in June and second in August to September. *Phlebotomus papatasi* (47.1%) was the most dominant species in out/indoors of both Shiraz (31.1%) and Kharameh (16.0%). It was also the only species which was found infected with *Leishmania major*, indeed, 2.68% and 2.53% of *P. papatasi* were infected to *L. major* in Kharameh and Shiraz, respectively. However, none of the female sand flies was positive for *Crithidia* spp. Despite various control strategies, especially against *Leishmania*, considerable cases of leishmaniasis are recorded from Iran every year. Phlebotomine plays the main role in transmission of *Leishmania* in these foci. Therefore, further studies are needed to determine the role of different phlebotomine species in epidemiological aspects of leishmaniasis.

Keywords Cutaneous leishmaniasis · *Phlebotomus papatasi* · *Leishmania* · *Crithidia* · PCR · Iran

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Introduction

Psychodids, known as "moth flies" or "drain flies", consist of more than 3000 described species and are distributed among six subfamilies of Phlebotominae, Bruchomyiinae, Sycoracinae, Horaiellinae, Psychodinae, and Trichomyiinae in the world (Bejarano and Estrada 2016). The adults are small (about 1–5 mm) and their hairy body and wings give them a "furry" moth-like figure (Schulz-Stübner et al. 2015). During their life cycle as a holometabolous insect, they live in aquatic, semiaquatic, or terrestrial ecosystems. The immatures grow and develop in different microhabitats

(the moist soil at the bases of trees, running or still bodies of wastewater or freshwater, drains of bathrooms, rotten tree trunks, kitchens, etc…). Adults prefer to rest in relatively humid microhabitats (caves, undersides of leaves, burrows, stables, interior walls of buildings, etc…) and tend to feed on plant materials (nectar). In addition, females of some species feed on blood of vertebrates, including humans (Young and Duncan 1994). The females of some species of Phlebotominae and Psychodinae subfamilies can transmit bacteria, viruses, or protozoan agents such as leishmaniasis that causes 20,000–40,000 deaths every year (Alvar et al. 2012). Besides, some species have been identified as the potential mechanical vectors of bacterial pathogens related to nosocomial infections (in some types of myiasis) or as the allergen sources of some asthma cases (Oğuz et al. 2012; Faulde and Spiesberger 2013).

Protozoan parasites of Trypanosomatidae have been identified with a single kinetoplast and flagellum and a mitochondrial DNA in the form of continued maxicircles and minicircles (Yurchenko and Kolesnikov 2001). They are divided into two groups of dixenous (two hosts) and monoxenous (one host). Two-host species are transmitted periodically between an insect vector and a plant or vertebrate host in their life cycle. In this group, *Leishmania* and *Trypanosoma* species are known to be pathogenic for humans and animals. They have been responsible for different diseases affecting more than 22 million people in the world yearly (Ishemgulova et al. 2017; Kalantari et al. 2018). *Leishmania* species, as obligatory-intracellular agents, invade and replicate within the macrophages in the immune system of humans and other hosts (Gholamian-Shahabad et al. 2018). The parasite has two different forms of promastigote (with flagellate) and amastigote (without flagellate), which occur in insect and mammalian hosts, respectively (Service and Ashford 2001). The monoxenous group species are pathogenic for arthropods, but not for humans. Unlike dixenous species that have been widely studied, the monoxenous species have remained neglected (Ishemgulova et al. 2017). As the monoxenous species are vital for tracking the development of parasitism and have an important effect on their hosts' physiological fitness and insects' communities in a global system, accurate investigations on their biology, host physiology, cellular biology, biochemistry, biodiversity, and genetics are unavoidable (Ishemgulova et al. 2017). One-host species have been recorded as co-infecting pathogens along with *Leishmania* species in immunocompetent and immunocompromised patients (Kraeva et al. 2015). *Crithidia* species, as the monoxenous species, exclusively parasitize arthropods, especially insects. They are identified with the presence of a free flagellum and the barley corn-like shapes (choanomastigote forms). Moreover, the digestive tracts of insects are the developing location of *Crithidia* cysts where they can easily move from one host to another by fecal–oral rout (Santos et al. 2006). *Crithidia* species have made relationships with other Trypanosomatidae parasites; some species of *Crithidia* have survived and have been co-detected along with some species of *Leishmania* in human and animal hosts (Santos et al. 2006; Doudi et al. 2015; Ghobakhloo et al. 2018; Kalantari et al. 2018).

Most known monoxenous species have been described based on morphology, life cycle, and host specificity (Votýpka et al. 2015). However, biochemical and molecular assays are needed and have been developed to classify or re-classify Trypanosomatids to provide accurate phylogenetic resolution of species (Frolov et al. 2016). For example, phylogenetic analyses on *Crithidia fasciculata*, as the type species of *Crithidia*, showed that many *Crithidia* species did not cluster with *C. fasciculata* and belonged to other genera of Trypanosomatidae (Yurchenko et al. 2008).

Various specific and sensitive polymerase chain reaction (PCR) detections of *Leishmania* kDNA have been used to confirm the presence of the parasite in vectors and hosts (Gholamian-Shahabad et al. 2018). Accurate detection assays are needed to distinguish *Leishmania* and *Crithidia* species from each other for proper treatment and suitable health control methods.

In Iran, *Leishmania major*, *L. tropica* and *L. infantum* (rarely) are involved in cutaneous leishmaniasis (CL) (Gholamian-Shahabad et al. 2018). CL is endemic in different parts of Iran, including Fars province. Indeed, there are some records regarding the presence of Crithidia-like organisms in *Leishmania* cultures in the laboratory and their possible survival in rodents and uncured old lesions from zoonotic CL (ZCL) patients of Shiraz and Kharameh (Ghobakhloo et al. 2018; Kalantari et al. 2018). This encouraged the authors to deplore the existence of *Leishmania* and *Crithidia* in sand fly vectors in these foci. Therefore, the present study aims to study the monthly activity, fauna, and detection of protozoan agents of *Leishmania* and *Crithidia* in phlebotomine sand flies using PCR in re-emerging ZCL foci of Shiraz and Kharameh (80 km from northeastern of Shiraz) in Fars province, southern Iran during 2016–2017.

Materials and methods

Study area

Fars province, with an area of 122,400 km^2, is situated in south of Islamic Republic of Iran, and includes 23 counties. Shiraz is the capital city of the province and Kharameh is placed about 80 km northeastern of Shiraz. Kharameh and Shiraz foci are located at 29°50′20″North, 53°31′24″East,

and 29°59′18″N, 52°58′37″E, and about 1500 and 1200 m above the sea level respectively. Newly, Shiraz and Kharameh are considered as the main important areas of zoonotic cutaneous leishmaniasis in Fars province, southern Iran (Fig. 1).

Sand flies collections

During the study on phlebotomine sand flies, 6975 sand flies of 16 species (eight *Phlebotomus* and eight *Sergentomyia* species) were caught from different regions of Shiraz and Kharameh foci from March 2016 to March 2017. Phlebotomine sand flies were caught using sticky paper traps from Abshoor, Ahmadabad, Helalabad, Kheirabad, Koohgari, Korbal, and Moezabad villages in Kharameh focus and Mahmoodabad, Sangesiah, Soltanabad, and Zafarabad villages in Shiraz focus (Fig. 1).

To determine the monthly activity of sand flies, they were caught from indoors and outdoors of both lowland and highland villages. For each collection, they were sampled from indoors, such as bedrooms and bathrooms, and outdoors, such as rocks, rodent burrows, agricultural lands, and walls gaps. In each sampling round, 60 sticky paper traps (30 indoors, 30 outdoors) were fixed in sunset and collected in the next morning. Phlebotomine sand flies were collected and kept in ethanol (70%), were mounted in the Puri's media, and were taxonomically identified according to valid taxonomic criteria studies (Theodor and Mesghali 1964; Lewis 1982). Afterward, all body segments of female phlebotomine sand flies (blood-fed and digested uniparous) were processed for DNA extraction and PCR assays.

PCR assays

DNA extraction

For DNA extraction, dissected sand flies (their thorax and anterior abdominal segments) were completely homogenized in ethanol (96%) with glass pestle. Afterwards, they were extracted by genomic DNA extraction mini kit instructions (Cat no. YT9030, YTA, Yekta Tajhiz Azma, Iran). Accordingly, for initial lyses, 200 µl of lysis buffer and 20 µl proteinase K were added to the samples, mixed, and incubated at 60 °C for 15 min. In continue, 200 µl ethanol (100%) was added to the mixtures, mixed for 30 s, transferred to column micro-tubes carefully, and centrifuged at 8000 rpm for 60 s. To remove impurities from micro-tubes, they were washed several times by washing buffers. Lastly, elution buffer (100–200 µl) was added to the membrane center of the tubes, centrifuged at 14,000 rpm for 2 min to elute the DNA after 3 min, and transferred to − 20 °C before PCR tests (Kalantari et al. 2018).

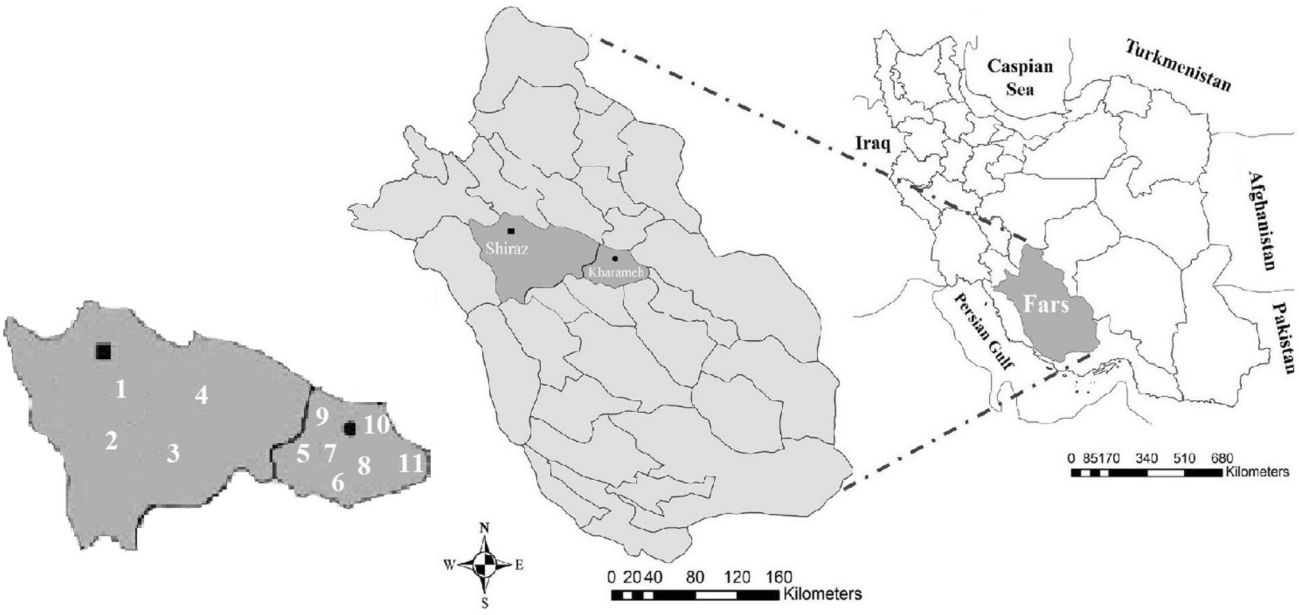

Fig. 1 Map of the studied areas of Shiraz and Kharameh in Fars province, southern Iran: Sangesiah (No. 1), Soltanabad (No. 2), Zafarabad (No. 3), and Mahmoodabad (No. 4) in Shiraz focus; Kheirabad (No. 5), Abshoor (No. 6), Koohgari (No. 7), Ahmadabad (8), Korbal (No. 9), Moezabad (No. 10), and Helalabad (No. 11) in Kharameh focus

Parasites detections

Leishmania

Female *Phlebotomus* species play the main role in human leishmaniasis transmission. Therefore, in vector studies, only females were checked for *Leishmania* detections and *Sergentomyia* species were removed from PCR investigation (Azizi et al. 2016a). *Leishmania* species were detected by a sensitive modified PCR assay (Azizi et al. 2017). Each 25-µl reaction mixture contained 3 µl DNA sample, 12 µl Ampliqon taq DNA polymerase master mix red, 1 µl (with 10 pico mol concentrations) of each primers of LIN17 (forward) (5′-TTT GAA CGG GAT TTC TG-3′) and LINR4 (reverse) (5′-GGG GTT GGT GTA AAA TAG GG-3′), and 8 µl double distilled water. For PCR, the thermocycler was programmed for one cycle of initial denaturation at 94 °C (for 5 min), continued for 30 cycles of denaturation at 94 °C (for 30 s), annealing at 52 °C (for 30 s), and extension at 72 °C (for 60 s), and finally involved one cycle of post denaturation at 72 °C (for 5 min). In continue, PCR products were run in electrophoresis gel (1.2%) stained with ethidium bromide and visualized with UV trans-illuminator. Reference strains of *L. major* (MHOM/IR/54/LV39) with a band of 650 bp would validate the presence of *L. major* kDNA (Azizi et al. 2016b).

Crithidia

Extracted DNAs of phlebotomin sendflies were selected using Internal Transcribed Spacer (ITS) gene for *Crithidia* minicircle kDNA sequences detections (Ghobakhloo et al. 2018; Kalantari et al. 2018). Therefore, for each PCR reaction, the total volume of 25-µl reaction mixture contained 5 µl DNA phlebotomine samples, 12 µl master mix buffer [Cat No. A180301, Ampliqon taq DNA polymerase master mix red (containing 1.5 mM $MgCl_2$ and 2× concentration of taq DNA polymerase)], 1 µl of each primer (concentrations of 10 pico mol) of 3′-CGCGTCGTTGATGAAGTCGCT-5′ and 5′-TCCATGTGCGAGGACAACGTGCT-3′, and 6 µl double distilled water. The samples were programmed and transferred to the thermocycler device (Eppendorf Master-cycler, Germany) for PCR implementation as follows: one cycle of initial denaturation at 94 °C (for 5 min), 30 cycles of denaturation at 94 °C (for 30 s), annealing at 55 °C (for 60 s), extension at 72 °C (for 1.5 min), and one cycle of final extension at 72 °C (for 5 min). In continue, 5 µl of PCR products were used in electrophoresis detection. The reference strain of *C. fasciculata* was used and a band of 800 bp would reveal the presence of *Crithidia* in the phlebotomine samples (Ghobakhloo et al. 2018).

Results

Sand flies compositions

Among the sand flies, 4048 (58.0%) were male and 2927 (42.0%) were female. In addition, the 5246 (75.2%) identified *Phlebotomus* species included 3288 *P. papatasi* (47.1%), 1078 *P. sergenti* (15.5%), 692 *P. alexandri* (9.9%), 164 *P. bergeroti* (2.3%), 16 *P. ansarii* (0.2%), 6 *P. major* (0.1%), 1 *P. mongolensis* (0.01%), and 1 *P. longiductus* (0.01%). On the other hand, the 1729 (24.8%) *Sergentomyia* species consisted of 521 *S. sintoni* (7.4%), 500 *S. baghdadis* (7.1%), 326 *S. theodori* (4.7%), 320 *S. antennata* (4.6%), 25 *S. clydei* (0.4%), 19 *S. squamipleuris* (0.3%), 11 *S. dentata* (0.2%), and 7 *S. mervynae* (0.1%) (Fig. 2).

The most dominant species were *Phlebotomus papatasi* (47.1%) collected from indoors and outdoors of both Shiraz (31.1%) and Kharameh (16.0%) foci. Also, the least abundant species were *P. mongolensis* and *P. longiductus* (each one 0.01%). Indeed, the two late species were just detected in Kharameh focus (Table 1).

In Shiraz focus, out of the 5123 samples, 2169 *P. papatasi*, 975 *P. sergenti*, 663 *P. alexandri*, 16 *P. ansarii*, 497 *S. sintoni*, 480 *S. baghdadis*, 305 *S. theodori*, 11 *S. dentata*, and 7 *S. mervynae* were collected from the central area, namely Sangesiah, and Mahmoodabad, Soltanabad, and Zafarabad villages as the suburban areas of the city (Table 1). *P. papatasi* was caught from all studied areas of Shiraz. However, some species, such as *P. sergenti*, *P. alexandri*, and *P. ansarii*, were only collected from Sangesiah, Soltanabad, and Zafarabad, respectively (Table 1). Among the other 1852 samples, 1119 *P. papatasi*, 164 *P. bergeroti*, 103 *P. sergenti*, 29 *P. alexandri*, 6 *P. major*, 1 *P. mongolensis*, 1 *P. longiductus*, 320 *S. antennata*, 24 *S.*

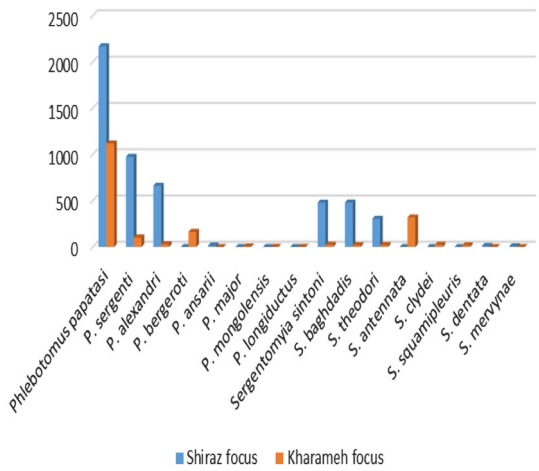

Fig. 2 Composition of naturally caught sand fly species in different areas of Shiraz and Kharameh in Fars province, southern Iran in 2016

Table 1 Abundance of sand fly species caught from outdoors and indoors of different areas of Shiraz and Kharameh in Fars province, southern Iran during 2016–2017

Genus and species	No. of collected Phlebotomine sand flies						
	Shiraz focus			Kharameh Focus			Total
	Males Outdoor/indoor	Females Out./In.	Total (M. & F.) Out./In.	Males Out./In.	Females Out./In.	Total (M. & F.) Out./In.	Sh./Kh.
Phlebotomus papatasi	846/416	671/236	1517/652	226/448	177/268	403/716	2169/1119
P. sergenti	388/198	266/123	654/321	41/25	21/16	62/41	975/103
P. alexandri	266/120	198/79	464/199	12/9	5/3	17/12	663/29
P. bergeroti	0/0	0/0	0/0	42/43	44/35	86/78	0/164
P. ansarii	6/4	2/4	8/8	0/0	0/0	0/0	16/0
P. major	0/0	0/0	0/0	1/0	5/0	6/0	0/6
P. mongolensis	0/0	0/0	0/0	1/0	0/0	1/0	0/1
P. longiductus	0/0	0/0	0/0	1/0	0/0	1/0	0/1
Sergentomyia sintoni	104/53	228/112	332/165	7/9	4/4	11/13	497/24
S. baghdadis	208/103	111/58	319/161	9/5	2/4	11/9	480/20
S. theodori	138/61	75/31	213/92	9/7	3/2	12/9	305/21
S. antennata	0/0	0/0	0/0	85/117	66/52	151/169	0/320
S. clydei	0/0	0/0	0/0	11/8	4/2	15/10	0/25
S. squamipleuris	0/0	0/0	0/0	4/3	7/5	11/8	0/19
S. dentata	6/4	0/1	6/5	0/0	0/0	0/0	11/0
S. mervynae	2/2	2/1	4/3	0/0	0/0	0/0	7/0
Total	1964/961	1553/645	3517/1606	449/674	338/391	787/1065	5123/1852
(%)	2925 (41.9)	2198 (31.5)	5123 (73.4)	1123 (16.1)	729 (10.5)	1852 (26.6)	6975 (100)

sintoni, 21 *S. theodori*, 20 *S. baghdadis*, 25 *S. clydei*, and 19 *S. squamipleuris* were caught from seven studied areas of Kharameh. In this context, *P. papatasi* was collected from all villages, but *P. bergeroti*, *P. major*, *P. mongolensis*, and *P. longiductus* species were merely collected from Ahmadabad, Koohgari, Helalabad, and Kheirabad, respectively (Table 1).

Sand flies activities

Sand flies activities started in early April in both Shiraz and Kharameh. In continue, two active peaks of sand flies were observed; the first occurred in early June and the second started in late August to early September in both foci. Phlebotomine activities terminated in late November and October in Shiraz and Kharameh, respectively (Fig. 3).

PCR outcomes

Considering the natural infection of the sand flies with *Leishmania* species, out of the 390 females including 299 *P. papatasi*, 43 *P. bergeroti*, 35 *P. sergenti*, 8 *P. alexandri*, and 5 *P. major* checked by PCR, 2.68% of *P. papatasi* (8/299) were positive for *L. major* in Kharameh focus. The

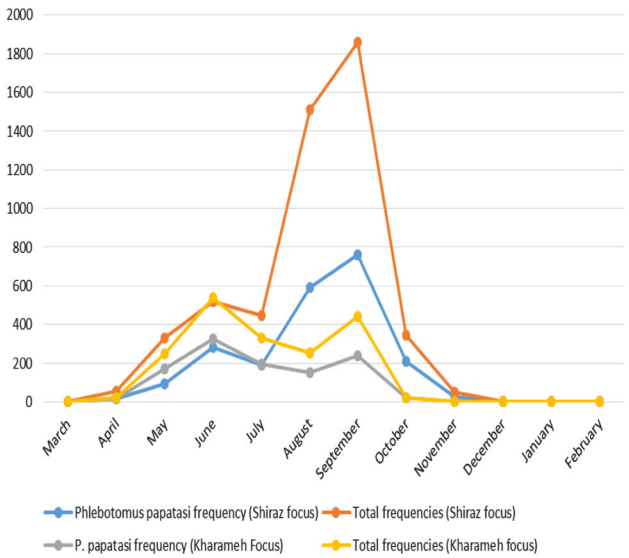

Fig. 3 Monthly activities of Phlebotoine sand flies in the studied areas of Shiraz and Kharameh, Fars province, southern Iran in 2016

infected sand flies were caught in Ahmadabad (2/145), Helalabad (1/10), Kheirabad (2/87), Koohgari (2/26), and Moezabad (1/31). Similarly, out of the 302 female *Phlebotomus* species (158 *P. papatasi*, 77 *P. sergenti*, 61 *P.*

alexandri, and 6 *P. ansarii*) tested in Shiraz focus, 2.53% of *P. papatasi* (4/158) were infected with *L. major* and belonged to Mahmoodabad (3/95) and Zafarabad (1/32) (Fig. 4). In the PCR test for *Crithidia* detections, none of the female samples of Phlebotomines was positive for *Crithidia* spp. parasites in both studied foci of Shiraz and Kharameh (Table 2).

Discussion

In Middle East regions, including Iran, *P. papatasi*, *P. sergenti*, *P.salehi*, *P. ansarii*, *P. kandelakii*, *P. caucasicus*, and *P. perfiliewi* have been reported as the proven or probable vectors of CL and Visceral Leishmaniasis (VL) (Killick-Kendrick 1999; Rassi et al. 2009). In south of Iran, *P. papatasi*, *P. major*, *P. keshishiani*, and *P. alexandri* have been reported as the vector(s) of CL and VL in different parts of Fars and Bushehr provinces (Azizi et al. 2006, 2008, 2010; Davami et al. 2011; Alipour et al. 2014). Fars is an important ZCL endemic focus and recent investigations revealed that the prevalence of the disease was remarkable (Azizi et al. 2016a).

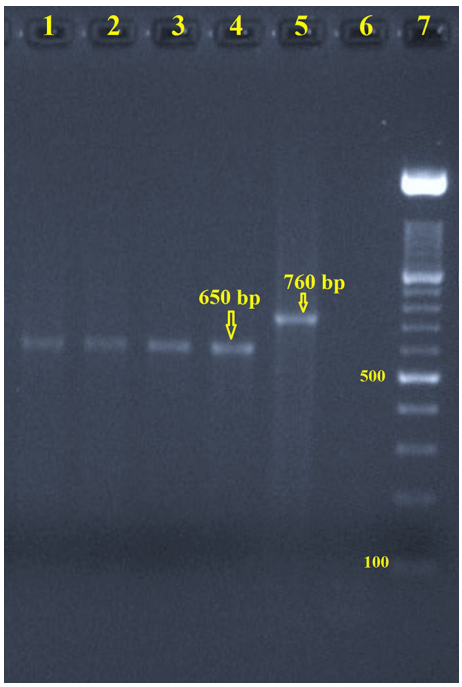

Fig. 4 Gel electrophoresis of PCR-based products of *Leishmania* detections. Samples prepared from the sand flies caught from Shiraz and Kharameh foci during 2016 and 2017; two female samples of *Phlebotomus papatasi* prepared from Kharameh focus (Lanes 1 and 2), female *P. papatasi* collected from Shiraz focus (3), reference strains of *Leishmania major* (650 bp) (Lane 4), reference strains of *L. tropica* (760 bp) (Lane 5), male sample of *P. papatasi* as the negative control (Lane 6), and the bands corresponding to the molecular weight marker (Lane 7)

In the current study, among the 6975 sand flies caught, eight (76.1%) *Phlebotomus* species and eight (23.9%) *Sergentomyia* species were identified. *P. papatasi* was the most dominant species and was frequently collected from in/outdoors of people's residual places in all studied regions of Shiraz and Kharameh. Outdoor breeding places of this species included agricultural lands where products, such as wheat, alfalfa, and corn, were planted. On the other hand, indoor places mostly included wall gaps of old bathrooms and bedrooms. The outcomes of the molecular assays revealed that 2.68% and 2.53% of *P. papatasi* were positive for *L. major* in Shiraz and Kharameh, respectively. This species is well distributed in the Old World and is naturally infected with *L. major* in different parts of Iran, including Fars province (Azizi et al. 2010).

Previously, *P. alexandri* was reported as the probable vector of *L. infantum* in Fars province (Azizi et al. 2006, 2008, 2010???). Furthermore, *P. sergenti* was recorded as the only proven vector of *L. tropica* in Iran (Aghaei Afshar et al. 2014). It has been found to be infected with the parasite in Shiraz and some other important cities in the country (Moin-Vaziri et al. 2007). In this study, these species were caught from both foci of Shiraz and Kharameh, but none of them was infected with *Leishmania* spp.

Phlebotomus major has been distributed in northern and southern parts of Iran, and natural infection of this species with *L. infantum* has been reported from Fars province (Azizi et al. 2008???). However, in the present study, this species was not infected with *Leishmania* spp. in the two studied areas.

Different molecular methods have been used for *leishmania* detection, but PCR assay is very helpful for *Leishmania*/*Crithidia* detections. However, in the current study, *Crithidia sp.* was not detected in sand flies. Yet, the presence and role of *Crithidia* in leishmaniasis infection in humans has remained considerable (Doudi et al. 2015; Ghobakhloo et al. 2018).

In conclusion, despite implementation of various control program strategies focused especially against *Leishmania* and malaria agents, new remarkable cases of vector-borne diseases are recorded from Iran every year (Gholamian-Shahabad et al. 2018; Ghahremani et al. 2014; Kalantari et al. 2017). In terms of CL, some diseases are anthroponotic form caused by *L. tropica*, but the common form of the disease is ZCL caused by *L. major* (Moemenbellah-fard et al. 2015). Therefore, considering the main role of sand flies in epidemiological aspects of ZCL, the results of the current investigation revealed that *P. papatasi* was the main vector in Kharameh and Shiraz foci. However, despite not finding any *Leishmania* infection among other caught phlebotomine sand flies in the studied areas, they might play secondary roles in transmission of CL agents in

Table 2 Number of female Phlebotomine sand flies checked for *Leishmania*/*Crithidia* detections by PCR assay in Shiraz and Kharameh foci during 2016–2017

Genus and species	No. of tested phlebotomine sandflies					
	Shiraz focus		Kharameh focus		Total	
	Females Checked	Infected Leishmania/Crithidia	Females Checked	Infected Leishmania/Crithidia	Females Checked	Infected Leishmania/Crithidia
Phlebotomus papatasi	158	4/0	299	8/0	457	12/0
P. sergenti	77	0/0	35	0/0	112	0/0
P. alexandri	61	0/0	8	0/0	69	0/0
P. bergeroti	0	0/0	43	0/0	43	0/0
P. ansarii	6	0/0	0	0/0	6	0/0
P. major	0	0/0	5	0/0	5	0/0
Total	302	4 (1.32%)/0	390	8 (2.05%)/0	692	12 (1.7%)/0

these foci. Besides, *Crithidia* was not detected in Iranian caught sand flies. Regarding ZCL epidemiology, further studies are needed to determine the role of *Crithidia* species along with *Leishmania* species.

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Authors contributions All authors contributed to the initial design, data collection, analyses, and manuscript writing. MK and KA wrote the proposal and designed the methods. MK and ZS collected the samples, identified the sand flies, and detected the parasites. KA, MHM, QA, and AS were involved in designing data screening and analysis. MK wrote the initial manuscript draft. All authors reviewed, revised, and confirmed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval In this study, ethical permission (No. IR.SUMS.-REC.1395.S475) was granted through the Science and Ethics Committee of Shiraz University of Medical Silences. All performed procedures were in accordance with the ethical standards of the Iranian institutional and/or national research committee and with the standards of 1964 Helsinki declaration.

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