Sandflies species composition, activity, and natural infection with *Leishmania*, parasite identity in lesion isolates of cutaneous leishmaniasis, central Iran

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Abstract Zoonotic cutaneous leishmaniasis (ZCL) is a crucial public health challenge in Iran. Sandflies feed on reservoir rodents' blood infected with *Leishmania* parasite and transmit it to other hosts. This study was conducted to find out the composition and monthly activity of sandflies as well as to identify the protozoan pathogens (*Leishmania*/*Critidia*) by polymerase chain reaction (PCR) in an emerging ZCL focus of Abarkooh, Yazd province, Iran, in 2016. A cross-sectional study was done in rural areas of Abarkooh. From April to November 2016, sticky traps

were used indoor and outdoor to capture sandflies once every fortnight. Their composition and monthly activity were recorded. Following identification of sandflies and DNA extraction from them, PCR was used to identify their parasite and match it against samples taken from ZCL confirmed and suspected patients' lesions. After collection, a total of 2045 sandflies (779 indoor, 1266 outdoor) were identified to species level. Sandfly activity started early April in this area with two active peaks (one late May and the other late August) terminated about mid-November. Seven *Phlebotomus* species and three *Sergentomyia* species were identified. The most and the least abundant species were *P. papatasi* (40.1%) and *P. alexandri* (0.09%), respectively. Using PCR, only 6% (12/200) of *P. papatasi* sandflies were infected with *Leishmania* parasite. No *Critidia* was detected in either sandflies or human lesions (176 specimen). Based on the highest abundance both indoor and outdoor of *P. papatasi*, this sandfly was considered the main vector of ZCL in this area. The capture of *P. caucasicus*, *P. mongolensis*, and *P. ansarii* from rodent burrows showed these species were likely involved in pathogen transmission in reservoir rodents' burrows.

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Introduction

Leishmaniasis is an important group of vector-borne infectious disease, caused by the parasitic protozoan, *Leishmania* (Kinetoplastida: Trypanosomatidae). It is present in 97 countries and about 350 million people are afflicted with this disease having symptomatic and asymptomatic individuals (World Health Organization

2018). It is transmissible by sandflies (Diptera: Psychodidae), *Phlebotomus*, in the Old World. Certain mammals such as canines and rodents act as reservoir hosts.

Leishmaniasis exhibit a wide range of clinical diseases including cutaneous leishmaniasis (CL). This is commoner than the other four (visceral, mucocutaneous, post-Kala Azar dermal, and diffuse CL) forms such that 1.5 million new CL cases are annually reported. More than 90% of such cases occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru (Alvar et al. 2012).

Leishmania is an obligatory intracellular pathogen which is involved in invasion, evasion, and replication within the host immune system macrophages. This flagellate parasite occurs in two different amastigote (no flagellate) and promastigote (flagellated) forms based on the ambient conditions in mammalian and insect hosts, respectively (Service and Ashford 2001). *Leishmania* is a dioxenous pathogen of sandflies and vertebrate hosts, whereas the closely-related genus *Crithidia* are normally monoxenous agents of insects (Molyneux and Ashford 1983). The latter parasites are easily cultivated and both genera occur in the subfamily Leishmaniinae (Akhoundi et al. 2016).

In Iran, *Leishmania major*, *Leishmania tropica* and rarely *Leishmania infantum* cause CL (Mohammadpour et al. 2017). Zoonotic CL (ZCL), induced by *L. major*, is endemic in all but less than half of the Iranian provinces and thus deserves special consideration. Recent reports on the probable contamination of *Leishmania* cultures in the laboratory with *Crithidia*-like organisms and their possible presence in refractory (uncured) old lesions from ZCL patients instigated the authors to deplore the presence of *Leishmania/Crithidia* in sandfly vectors and human lesions (Doudi et al. 2014).

Molecular techniques are increasingly used to confirm *Leishmania* kDNA (Kinetoplast DNA) presence in vectors and hosts. The detection of flagellate parasite kDNA by polymerase chain reaction (PCR) method is very specific and sensitive, with efficacies summing up to 100% (Bensoussan et al. 2006). Accurate identification methods are needed to distinguish *Leishmania/Crithidia* species from each other, which is essential in proper treatment and appropriate health control measures.

To the best of authors' knowledge, there has been no documented evidence on the presence of flagellate protozoa in sand fly vectors and human lesions in this part of Iran so far. The main aim of this study was to investigate the composition and monthly activity of sandflies as well as to identify protozoan agents (*Leishmania/Crithidia*) using polymerase chain reaction (PCR) from *Phlebotomus papatasi* sandfly and species identity in lesions of cutaneous leishmaniasis in an emerging ZCL focus of Abarkooh, Yazd province, Iran, in 2016.

Materials and Methods

Study area

This cross-sectional descriptive study was implemented from April to November 2016 in Abarkooh. This area ($\approx 5641 \text{ km}^2$) lies on the western part of Yazd province in center of Iran (Fig. 1). It has a desert plain ($46^\circ 30' \text{N}$, $53^\circ 28' \text{E}$) with an altitude of 1510 m above sea level. Sandfly samples were gathered from seven (three stable, four variable) villages across Abarkooh. These included Hosseinabad, Nosratabad, Asadabad-Esfandabad; and Faragheh, Chahgir, Gonbad, and Esfandabad.

Abarkooh area is located between Fars and Isfahan provinces where ZCL is endemic (Azizi et al. 2012a). It has a hot and dry climate, and most settlements are made up of mud and corrugated bricks. People endeavor in agricultural activities, frequently keep hens or ungulates in their houses and may stay or rest outside for long periods. Rodent colonies are also abound near human premises (Najafzadeh et al. 2014). Due to its vicinity to ZCL-endemic provinces of Fars and Isfahan (Khosravani et al. 2016), Abarkooh is regarded as an emerging new ZCL focus.

Sandfly collection and species identification

Sandflies were caught using sticky paper traps from seven named villages of Abarkooh once every fortnight. For each collection, sandflies were sampled from indoors (bedroom, bathroom, etc.) and outdoors (rodent burrows, crevices in walls, rocks, etc.). In each sampling round, 60 sticky traps

Fig. 1 Map of the study area, Abarkooh, in Yazd province of Iran. The two central and Bahman districts included the three constant (1-Hosseinabad, 2-Nosratabad, 3-Asadabad-Esfandabad) and four variable (4-Gonbad, 5-Esfandabad, 6-Chahgir, 7-Faragheh) stations

(30 indoors, 30 outdoors) were fixed following sunset and removed every morning after. Each trap fixed on one night was taken as a “trap-night” and there were 120 “trap-nights”/month. Male sandflies were removed using acetone and fine entomology needles and kept in 70% ethanol for mounting and taxonomic studies, while females were processed for kDNA extraction.

The head and last two abdominal segments from each female sandfly were rinsed following dissection using two needles in a drop of Puri's medium on a microscope slide to determine species identity of each sandfly according to valid taxonomic keys (Lewis 1982; Theodor and Mesghali 1964). The remaining body segments of previously blood-fed and digested (void) uniparous females of the common phlebotomine sandfly species (*P. papatasi*, *P. sergenti*, etc.) were used for DNA extraction and later PCR processing. Parous female sandflies were distinguished from nulliparous ones by the presence or absence of yellow granules in the accessory glands. In contrast, nulliparous females had a well-developed volume of fat bodies which was reflected in the specific gravity (Molyneux and Ashford 1983). Thus, young female sandflies floated in saline solution in which older ones sank.

DNA extraction

Sandflies

DNA extraction from sandflies was carried out with the nested PCR method as reported before (Azizi et al. 2012a). First, the thorax and anterior abdominal segments of dissected sandflies were ground and homogenized in 96% ethanol within 1.5 mL Eppendorf® microtubes with fine glass pestle.

Human lesion smears

Slide smears (#176) from CL patients (#100) were scraped off with 200 µL TG1 buffer solution and the products were transferred to 2.5 µL microtubes. They were then extracted using the YTA genomic DNA extraction mini kit (Yekta Tajhiz Azma, Tehran, Iran).

Leishmania

The PCR used to amplify the variable area of the minicircle kDNA of any *Leishmania* in the sandfly and lesion smear was a slight modification of that described by Aransay et al. (2000). The forward LINR4 (5'-GGG GTT GGT GTA AAA TAG GG-3') and reverse LIN17 (5'TTT GAA CGG GAT TTC TG-3') primers used contain the conserved sequence blocks 3 and 2, respectively. Each 25-mL reaction mixture contained 250 mM of each deoxynucleoside

triphosphate, 1.5 mM MgCl₂, 1 U *Taq* polymerase (Cina-gene, Tehran), 1 mM LINR4, 1 mM LIN17 and 5 mL DNA extract, in 16 PCR buffer (Boehringer Mannheim, Mannheim, Germany). Each reaction mixture was overlaid with mineral oil before being transferred to a CG1-96 thermo cycler (Corbett Research, Sydney, Australia) set to give 5 min at 94 °C, followed by 30 cycles, each of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and then a final extension at 72 °C for 5 min. A 5 mL sample of each PCR product was subjected to electrophoresis in 1.2%-agarose gel. The bands were then stained with ethidium bromide and visualized under ultra-violet trans-illumination.

Crithidia

The rest of the stages were done according to kit's instruction (high pure PCR template preparation kit, Roche). Finally, DNA was dissolved in 200 µL of elution buffer. The extracted DNA was studied quantitatively and qualitatively by spectrophotometry and then agarose gel 0.8% in TBE 1 × buffer.

Amplification of ITS1 using PCR

Amplification of ITS1 using PCR was conducted through the LITSr primers L5.8S with the following sequences: LITSr (5'-CTGGATCATTTCCTCGATG-3') L5.8s (5'-TGATACCACTTATCGCACTT-3'). The best condition to implement PCR was determined by using the standard strains. The reaction was performed in 25 µL. First, a master mix was prepared which consisted of 0.2 µL of *Taq* polymerase (50 Mm), 2 µL MgCl₂, R and F primers with concentration of 10 pmol 1 µL of each, 2 µL PCR10× buffer, dNTPs (10 Mm) for 1 µL, and finally 1 µL extracted DNA was added to the above mentioned items, and the final volume of the distilled water reached 25 µL. The samples placed into a thermo cycler device (Corbett, Australia) and the following cycles were performed for the PCR implementation. Thermal cycle used for amplification of the required fragment through PCR method involved initial denaturation at 95 °C, 5 min, 1 cycle, denaturation at 95 °C, 20 s, 30 cycles, annealing at 52 °C, 30 s, extension at 72 °C, 1 min, and post extension at 72 °C, 6 min, 1 cycle.

Thereafter, the PCR products with positive and negative control along with molecular marker of 50 bp were placed on 1.5% agarose gel containing 0.7 µg/mL ethidium bromide per mL of gel in TBE 1 × buffer and then were electrophoresed for 25–45 min in a voltage of 80 V. The PCR products were observed in trans-illuminator device using UV lamp.

Detection and identification of *Leishmania* species

In order to detect the parasite genus, 5 mL of final products were subjected to electrophoresis on 1.5% (V/V) agar gel stained with ethidium bromide and observed by UV transillumination. The size of each band was compared with the standard strains of *Crithidia* and *Leishmania*. Parasites were identified by comparing the size of the band produced from a test sample with those produced from reference strains of *L. infantum* (MCAN/IR/96/LON49), *L. tropica* (MHOM/IR/89/ARA2) and *L. major* (MHOM/IR/54/LV39). A band of 560 bp reflected that *L. major* kDNA was present.

Statistical analysis

The collected data were analyzed using descriptive statistics and Chi squared test in SPSS V16 software. A *P* value of < 0.05 was considered to be significant.

Results

Some 2045 sandflies including seven (76.1%) species of *Phlebotomus* and three (23.9%) species of *Sergentomyia* were identified (Table 1, Fig. 2). Sandfly activity started early April in this area and with two active peaks (one late May and the other late August) (Fig. 3) terminated about mid-November. The most and the least abundant species were *Phlebotomus papatasi* (40.1%) and *P. alexandri* (0.09%), respectively. The former species is thus considered the dominant species in this area. The seven identified *Phlebotomus* species included: *P. papatasi*, *P. ansarii*, *P. caucasicus*, *P. mongolensis*, *P. salehi*, *P. alexandri*, and *P. sergenti*, whereas the three *Sergentomyia* species were *S. sintoni*, *S. antennata*, and *S. baghdadis*. In addition, *P.*

Fig. 2 Sandfly species composition naturally caught in Abarkooh, Iran

Fig. 3 Monthly activity of *Phlebotomus papatasi* in the study area, Abarkooh

papatasi was the dominant species both indoors (50.32%) and outdoors (33.8%), and *S. sintoni* (28.9%) was the most frequent species in outdoor-based observations of monthly activity study (Table 1). The peak of activity of this sandfly species was also twice a year (2nd half of June and early August).

Table 1 Fauna and abundances of different sandfly species caught in Abarkooh of Yazd province, Iran, 2016

Sandfly species/place	Indoor (%)	Outdoor (%)	Total (%)
<i>P. papatasi</i>	392 (50.32)	428 (33.8)	820 (40.1)
<i>P. ansari</i>	209 (26.82)	218 (17.22)	430 (21.02)
<i>P. caucasicus</i>	64 (8.2)	149 (11.8)	210 (10.3)
<i>P. sergenti</i>	8 (1.03)	41 (3.24)	49 (2.4)
<i>P. mongolensis</i>	0 (0)	25 (2)	25 (1.2)
<i>P. salehi</i>	1 (0.13)	19 (1.5)	20 (0.98)
<i>P. alexandri</i>	0 (0)	2 (0.16)	2 (0.09)
<i>S. sintoni</i>	105 (13.5)	366 (28.9)	471 (23.03)
<i>S. antennata</i>	0 (0)	17 (1.3)	17 (0.83)
<i>S. baghdadis</i>	0 (0)	1 (0.08)	1 (0.05)
Sum	779 (100)	1266 (100)	2045 (100)

The kinetoplast DNA of the parasite *L. major* was detected in 12 (6%) out of the 200 female phlebotomine sandflies with nested PCR (Table 2). None of the other 76 sandflies were positive with *L. major* kDNA. Likewise, no sandfly species were infected with *Crithidia* kDNA using standard PCR.

From 100 CL patients, 176 slide smears were taken from which half (52%) of them were positive with *L. major* kDNA using nested PCR (Figs. 4, 5), and none were representative of *Crithidia* kDNA presence using standard PCR in this area.

Discussion

The present data indicated that although ten different sandfly species were present in the studied area, only the main proven vector of ZCL (i.e. *P. papatasi*) was involved in pathogen passage, since it was the only sandfly species found to be positive with *L. major* kDNA. This assertion is a critical but insufficient condition to vector incrimination (Killick-Kendrick 1999). The closely-related species, *P. salehi*, was also mainly trapped outdoor and subjected to PCR assays, but it had no *L. major* kDNA. This is interesting since previous reports had indicated a role for this sandfly species in ZCL transmission (Azizi et al. 2012b; Davami et al. 2011). It may be a main vector among rodents.

Despite control programs, directed mainly against malaria vectors (Moemenbellah-Fard et al. 2012), more than 20,000 cases of CL are annually reported in Iran (Khosravani et al. 2016), and new disease foci continue to be discovered. Some of these may be the dry anthroponotic form of CL caused by *Leishmania tropica* (Moemenbellah-Fard et al. 2015) but the majority of cases are due to ZCL (Moemenbellah-Fard et al. 2003). Although the common vector of *L. tropica*, sandfly *P. sergenti* species, was also found to exist in the present study, but none of them were infected with any parasite. This research was the first on ZCL vectors in this study area. Based on these data,

Fig. 4 Gel electrophoresis from human lesion smears indicating a 650 bp band corresponding to standard *L. major* (lane 2), and human lesion smears (lanes 3–8)

P. papatasi was the most frequent species both indoors and outdoors, and it is thus regarded as the dominant sandfly species (Azizi et al. 2012c). This sandfly status was also confirmed in other studies (Alipour et al 2014; Azizi et al. 2012d, 2013). This sandfly activity pattern conforms to the trends in numbers of human active lesions.

Molecular (PCR) method was conducted to detect the presence of parasitic protozoa in sandflies and human

Table 2 Number and percentages of female sandflies tested with PCR assay

Genus	Total captured female sandflies	Tested female sandflies		Infected female sandflies	
		Number	%	Number	%
<i>P. papatasi</i>	505	200	23.23	12	6
<i>P. sergenti</i>	22	22	100	0	0
<i>P. salehi</i>	18	18	100	0	0
<i>P. caucasicus</i>	50	30	60	0	0
<i>P. mongolensis</i>	6	6	100	0	0
Total	601	276	26.75	12	6

Fig. 5 Gel electrophoresis from female sandflies showing lane 1 as the marker column, lanes 2–15 and 18–21 *P. papatasi* sandflies, lane 16 negative control, lane 17 positive control

lesions. Only *Leishmania major* was detected in both samples. This method was used since it was quite easy to compare with other methods in the laboratory, and numerous species could be tested in a short time. This method is, however, unable to distinguish between the promastigote and amastigote forms of the parasite.

The detection of *L. major* kDNA in wild sandflies of this region points to the increasing clinical significance of ZCL in recent years, in addition to the non-infectious (Ebrahimi et al. 2017) and other infectious diseases (Fakoorziba et al. 2006; Farhadpour et al. 2016; Neghab et al. 2006) in southern parts of Iran. Despite attempts to look for the presence of *Criethidia* parasite both in human lesion smears and naturally caught sandflies near rodent burrows and domestic settlements, no trace of its kDNA was discernible. This finding is consistent with the fact that *Criethidia* is a common monoxenous parasite of insects, while *Leishmania* is dixenous shifting between invertebrate and vertebrate hosts (Molyneux and Ashford 1983). The present study is in sharp contrast to other studies from Iran (Doudi et al. 2014; Ghobakhloo et al. 2018).

Conclusion

It is concluded that *P. papatasi* is the primary and main vector of *L. major* and it has no role of transmitting *Criethidia* parasite in this area. The identity of *L. major* from this sandfly species was in consistence with that from human lesions.

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Authors Contributions All authors contributed to different extents in the initial design, data collection, analyses and manuscript writing. The first author, MRG-S, wrote the proposal with its designed method, collected samples, identified them, implemented the PCR method, and collated all his findings in an MSc thesis. MRG-S wrote the initial MS draft. KA, QA, and MDM-F were involved in the design, data screening, data analysis, and MS draft preparation. MK and MDM-F were also accountable in the technical assistance of molecular studies and final draft writing.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of Science and Ethics Committee of SUMS University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical permission was granted through the Science and Ethics Committee of SUMS University. Informed consent was obtained from each individual's family in this study.

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