Original Article

Molecular Detection of *Leishmania* Infection in Phlebotomine Sand Flies from an Endemic Focus of Zoonotic Cutaneous Leishmaniasis in Iran

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Abstract

Background: Due to the outbreak of zoonotic cutaneous leishmaniasis (ZCL), a disease caused by *Leishmania major* and mainly transmitted by *Phlebotomus papatasi*, in Damghan City, Semnan Province, the probable vectors of the disease were investigated in the city from 20 March 2016 to 20 January 2018.

Methods: Sand flies were collected from indoors and outdoors biweekly by sticky traps in different parts of the city. The trapped sand flies were stored in 70% ethanol. They were identified and checked for *Leishmania* infections using nested-PCR method and specific primers; CSB1XR, CSB2XF, LiR, and 13Z.

Results: Overall, 1862 phlebotomine sand flies of *Ph. papatasi* (48.8%), *Ph. andrejevi* (8.3%), *Ph. caucasicus* (7.7), *Ph. mongolensis* (2%), *Ph. sergenti* (1.2%), *Ph. alexandri* (0.7%), *Sergentomyia murgabiensis sintoni* (29.3%), and *Se. sumbarica* (2%) were collected indoors (31.1%) and outdoors (68.9%). The highest and lowest numbers of collected sand flies were belonging to *Ph. papatasi* (48.8%) and *Ph. alexandri* (0.7%) respectively. 2.2% of the examined sand flies were shown to be infected with *L. major* and all were belonging to *Ph. papatasi*.

Conclusion: This study confirms the report of *Ph. papatasi* infection with *L. major* and also the existence of *Ph. sergenti* and *Ph. alexandri*, the potential vectors of *L. tropica* and *L. infantum* respectively, in Damghan City. According to the findings, it is necessary for health officials to plan and take action to prevent the occurrence of ZCL epidemic in the city as well as the occurrence of other forms of leishmaniasis.

Keywords: Molecular survey; *Leishmania major*; Sand fly; Nested PCR; Damghan

Introduction

Leishmaniasis, a protozoan parasitic infectious disease transmissible by the bite of the subfamily phlebotomine (Diptera: Psychodidae) sand flies and classified in the seventeenth neglected tropical diseases (NTD), occurs in tropical and subtropical areas of some 98 countries of the world (1). More than 350 million people are living in areas at risk of leishmaniasis and an estimated 2 million new cases of the disease occur annually. Distribution and incidence of leishmaniasis is not the same in endemic areas. Seven countries; Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria have about 90% of cutaneous leishmaniasis (CL) cases (2).

Two clinical types of cutaneous leishmaniasis including anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) with causative agents of *Leishmania tropica* and *L. major* respectively, have been reported from several parts of Iran (3, 4). Cutaneous leishmaniasis is the main vector-borne disease in Iran with an annual average of more than 22,000 cases that about 80% of

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them are zoonotic form. The endemic foci of this type of the disease are in rural areas of 18 out of 31 provinces (5, 6). Incidence of CL in Damghan District had been reported around 111 per 100,000 in 2009 (7).

There are more than 800 phlebotomine sand fly species, but 166 species are proven or suspected vectors of human leishmaniasis in the world. Furthermore, about 50 species of them have been found to be naturally infected with *Leishmania* species and have been introduced as the vectors of leishmaniasis (8-10). Gerbil rodents (Muridae: Gerbillinae) are the main reservoir hosts of ZCL in the most areas of Iran (5, 11, 12) including Damghan District (13, 14).

Iranian sand flies includes 48 species in two genera of *Phlebotomus* and *Sergentomyia*. Each genus has 6 subgenera in the Country (15). The subgenus *Sergentomyia* have no role in the transmission of leishmaniasis to human. They are vectors of lizard leishmaniasis. *Phlebotomus papatasi* is the main vector of ZCL, while *Ph. salehi* is also found to be infected with *L. major* in some foci of the disease. *Phlebotomus salehi* is mainly reported in the southern part of the country. *Phlebotomus ansari*, *Ph. salehi* and *Ph. caucasicus* group are known to have a role in zoonotic cycle of CL between rodents (5, 16). *Phlebotomus alexandri* have been reported in the most studies on sand flies in Iran and it is found to be infected with *L. infantum* (17).

Damghan District in in Semnan Province, northern Iran. It is situated 342km east of Tehran on the high-road to Mashad, at an elevation of 1,250m above sea level. At the 2016 census, the County's population was 94,190 in 30,296 households. This County is an important focus of ZCL, with annually more than 200 new cases of the disease in the last years (18). An outbreak of the disease, with about 1500 cases, has occurred in the rural areas of Damghan District in 1999 (18). Rodents such as *Rhombomys opimus*, *Nesokia indica* and *Meriones libycus* have been reported as the main reservoir hosts (14, 19) and *Ph. papatasi* has been introduced as the main vector of the disease in rural areas of the district (20).

At the beginning of the disease outbreak, leishmaniasis was only limited to the rural areas, but in recent years, ZCL agents were detected in the patients who had previously traveled to the endemic rural areas of Damghan (7, 18). This finding reinforces the hypothesis that the disease is spreading to the city and that there is a need to consider the necessary measures to prevent and control the disease. For vector control strategies, it was necessary to detect the *leishmania* parasites in the phlebotomine sand flies as the vectors of ZCL in Damghan focus. Therefore, this study was designed and conducted to identify the vectors (sand fly species) and their infection with *Leishmania* parasites in the city.

Materials and Methods

Study area

This descriptive cross-sectional study was conducted during 20 March 2016 to 20 January 2018 in different parts of Damghan City, in Semnan Province. Damghan is located in 1170m above sea level and 350km east of the capital, Tehran, in northern part of Iran (13).

The city is situated between Shahroud and Semnan Cities and is bounded on the mountainous areas in the north (Alborz Mountains) with 9.8 ℃ and the plain areas in the south (Kaveer Desert) with 23.5 ℃ average temperatures. The annual rainfall and average temperature of the city are about 120mm and 16.3 ℃ respectively (21). The city has generally a warm weather in summer and cold in winter.

Sand fly collection

Sand flies were collected from indoors (bedroom, guestroom, toilet and stable) and outdoors (e.g. rodent burrows and wall cracks) biweekly by sticky traps in different parts of the city. In each sampling, 300 sticky paper traps (30 indoors and 30 outdoors) were fixed in the sunset and collected in the next morning before sunrise. Phlebotomine sand flies were collect-

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ed and kept in ethanol (70%). The head and last abdominal segments of female sand flies were removed and mounted on a microscope slide in a drop of Puri's medium, and taxonomically identified according to valid taxonomic criteria (22). Each sample identified at species level, according to the standard identification keys (23, 24). The remaining portion of the female sand flies′ bodies were subjected to DNA extraction and molecular detection of *Leishmania* infection.

Molecular identification

Totally 179 samples of the female sand flies were selected for molecular detection of *leishmania* infection. Each female sample was separately checked for *Leishmania* infection by nested-PCR assay using specific primers (25).

DNA Extraction

Each sample were homogenized in a mixture of 200µl lysis buffer (50µl Tris-HCl [pH 7.6], 1% Tween 20 and 1µl EDTA), 12µl of proteinase K solution (19µl of the enzyme/ ml), in a 1.5ml sterile microcentrifuge tube. The homogenate incubated at 37 ℃ overnight, and then 300µl phenol: chloroform: isoamyl alcohol mixture (25:24:1, by vol.) were added. After shaking vigorously, the mixture was centrifuged (10,000 RPM for 10min). The extracted DNA in the supernatant was precipitated with 400µl of cold and pure ethanol, resuspended in 50µl of double-distilled water, and stored at -20 °C until using for detection of *Leishmania* parasites′ kDNA.

Amplification of Kinetoplast Minicircle DNA

Nested PCR assay was carried out in two rounds using the CSB1XR (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB2XF (CGA GTA GCA GAA ACT CCC GTT CA) primers for the first round and LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) for the second round. First, a total reaction mixture (25µl) was prepared which contained 5µl of template DNA, 12µl Master mix (containing deoxynucleoside triphosphate (Sinaclon, Tehran, Iran), Taq polymerase, MgCl2, Tris-HCl (pH 7.6)), 1µl of CSB1XR and 1µl of CSB2XF, and 6µl of Doubled Distilled Water (DDW). PCR reaction protocol was set in a thermocycler (Eppendorf AG; Humbug, Germany). The temperature program was set at 94 °C for 5min for the first extension, followed by 30 cycle (which was repeated at 94 °C for 30s, 55 °C for 1min, and 72 °C for 1.5min), and then a final extension at 72 ° C for 10min. For the second round, 1µl of the first-round products' dilution (1:9, by vol.) was used as the templates. The reaction for the second round was the same as the first round with an exception in the volume of the reaction mixture changed to 30µl (as the DDW volume was changed from 6µl to 11µl for the second round), and the use of 13Z and LiR primers. Finally, 5µl of the final products were run on 1.5% (V/V) agarose gel marked with ethidium bromide and visualized by ultraviolet trans-illumination. The size of bands was estimated by comparison with the size of the reference strains (12, 25).

Reference strains of *L. major* (MHOM/IR/ 54/LV 39), *L. infantum* (MCAN/IR/96/Lon 46) and *L. tropica* (MHOM/IR/89/ARD 2) were used as standards from Department of Medical Parasitology, Shiraz University of Medical Sciences. Also, double-distilled water was included in each run as a negative control. A band of 560bp, 680bp, and 750bp indicated the presence of *L. major*, *L. infantum* and *L. tropica* respectively.

Results

Totally, 1862 sandflies (822 females and 1040 males) including 8 species (six *Phlebotomus spp*. and two *Sergentomyia spp.*) were trapped and identified in a period of 22 months. They were belonging to; *Ph. papatasi* (48.8%), *Ph. mongolensis* (2%), *Ph. caucasicus* (7.7%), *Ph. sergenti* (1.2%), *Ph. alexandri* (0.7%), *Ph. andrejevi* (8.3%), *Se. murgabiensis sintoni* (29.3 %) and *Se. sumbarica* (2%) (Table 1).

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The sand flies′ sex ratio (ratio of male to female) was 126.5/100. Totally, 580 (31.1%) and 1282 (68.9%) specimens were collected from the indoor and the outdoor places respectively. 43.2% of the sandflies collected from the outdoors and 61% from the indoors were belonging to *P. papatasi*. 52.3 percent of the abdominal status of sand flies were empty, and the others were blood fed (10.3%), gravid (16%), and semi gravid (21.4 %) (Table 1).

Four (2.2%) of the 179 molecularly examined sand flies were shown to be infected with *L. major* parasites with band size about 560bp (figure 1). Of which, one was from indoor, 2 from rodent barrows and another one was from outdoor. 75% (3/4) of infected sandflies were collected in August and September, but 25% (1/4) were trapped in June.

Table 1. Details of sand flies collected in Damghan City, 2016–2018

Species	Total No. (%)	Male No. (%)	Female No. (%)	Abdominal status No. (%)				Indoor (%)	Outdoor (%)
				Blood fed	Empty	Semi-gravid	Gravid		
Phlebotomus papatasi	908 (48.8)	540 (59.5)	368 (40.5)	50 (13.6)	147 (40)	75 (20.4)	96 (26)	354(39)	**554 (61)**
Phlebotomus mongolensis	37 (2)	29 (78.4)	8 (21.6)	2 (25)	3 (37.5)	2 (25)	1 (12.5)	2(5.4)	**35 (94.6)**
Phlebotomus caucasicus	143 (7.7)	116 (81.1)	27 (18.9)	6 (22.2)	11 (40.7)	7 (26)	3 (11.1)	21(14.7)	**122 (85.3)**
Phlebotomus alexandri	12 (0.7)	10 (83.3)	2 (16.7)	-	2 (100)	-	-	-	**12 (100)**
Phlebotomus sergenti	22 (1.2)	18 (81.8)	4 (18.2)	-	4 (100)	-	-	7(31.8)	**15 (68.2)**
Phlebotomus andrejevi	156 (8.3)	126 (80.8)	30 (19.2)	5 (16.7)	12 (40)	8 (26.6)	5 (16.7)	14(9)	**142 (91)**
Sergentomyia murgabiensis sintoni	546 (29.3)	185 (33.9)	361 (66.1)	18 (5)	235 (65.1)	82 (22.7)	26 (7.2)	182(33.3)	**364 (66.7)**
Sergentomyia sumbarica	38 (2)	16 (42.1)	22 (57.9)	3 (13.7)	16 (72.8)	2 (9)	1 (4.5)	-	**38 (100)**
Total	1862	1040 (55.9)	822 (44.1)	84 (10.3)	430 (52.3)	176 (21.4)	132 (16)	580 (31.1)	**1282 (68.9)**

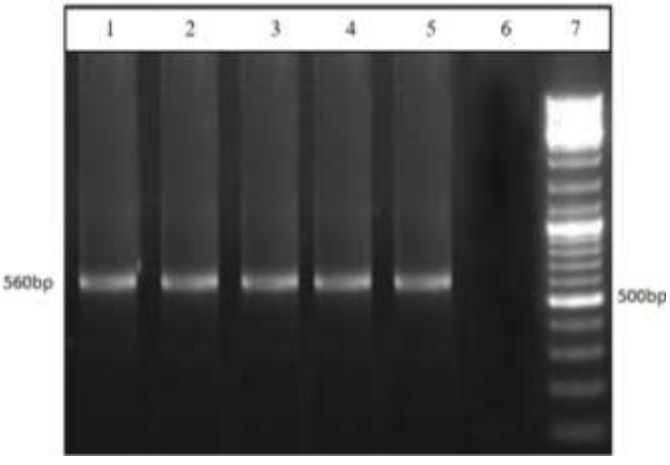

Fig. 1. Gel electrophoresis of Nested-PCR products of *Leishmania major* kDNA in *Phlebotomus papatasi* caught from urban areas of Damghan, 2016–2018. The wells correspond to *L. major* in *Ph. papatasi* (1-4), Reference strains of *L. major* (5), Negative control (DW) (6), and molecular weight marker (7)

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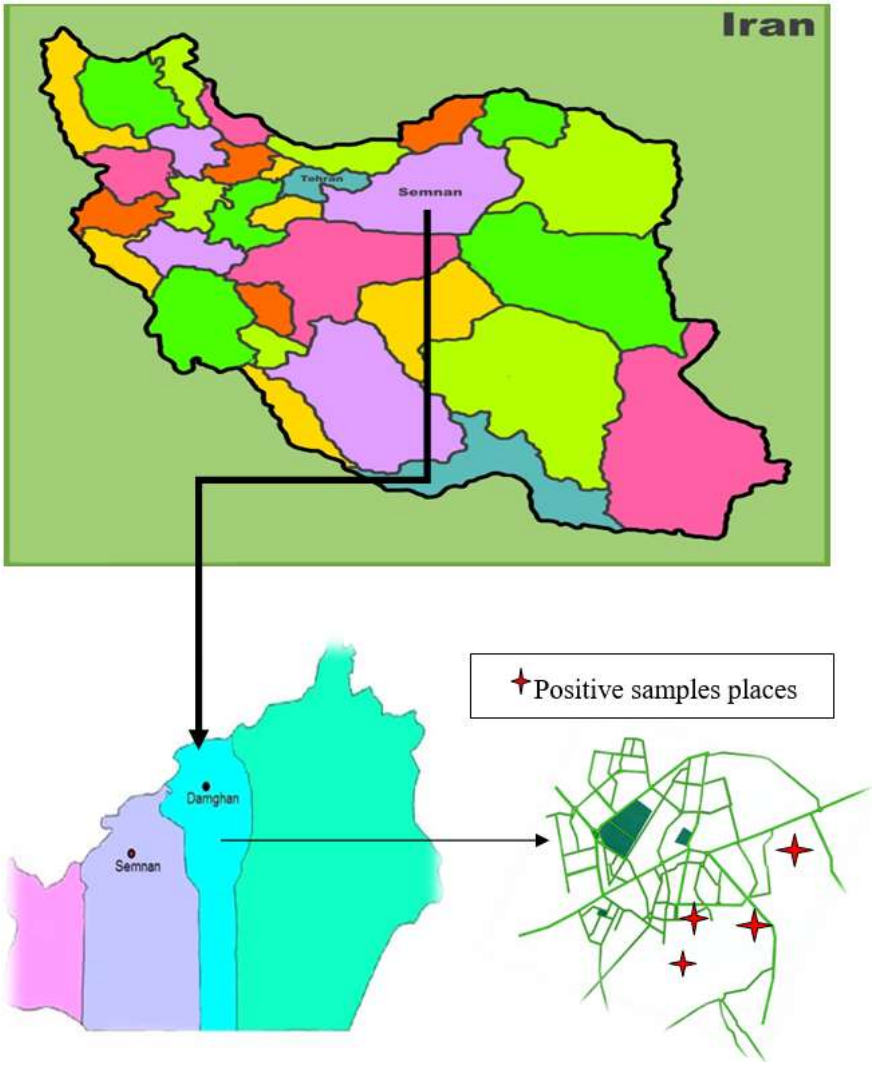

Fig. 2. Map of the distribution of *Leishmania major* infected sand flies in Damghan City, Semnan Province, Iran

Discussion

The identification of eight sandflies species in this study showed that these insects have a relatively high diversity in the city. So far, 44 species of both genera *Phlebotomus* and *Sergentomyia* of sand flies have been identified and reported in Iran (5, 20). Only four of the Iranian sand fly species were shown to be infected with *leishmania* parasites. Promastigotes of *leishmania* parasites have been found and reported in 13 various species of these significant biologic vectors from endemic foci of cutaneous and visceral leishmaniasis (5).

In the present study *Ph. papatasi* was dominant species and had the highest abundance in indoor and outdoor places and in rodent burrows. This species of sand flies, in most other studies, has also shown the highest frequency and they have been collected and reported from various parts of the country with an eight to 1756m altitude range (5). The study area, Damghan City, with an altitude of 1170m, arid and semi- arid climate is a suitable place for this species to survive. Besides climatic situations, some other factors such as the construction of new buildings and accumulation

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of their wastes in different parts of the city, the presence of reservoir rodents in those places may affect the spread of the disease. *Phlebotomus papatasi* is spread in the arid and semi-arid zones of Mediterranean, Europe, North Africa, Middle East, and the Indian subcontinent (11, 26-28) and it has been reported as a dominant species in other cutaneous leishmaniasis foci in Iran, notably in rural areas of the country and has been introduced as proven vector of ZCL (11, 29-33).

Among the molecularly examined sandflies, only the species of *Ph. papatasi* were shown to be infected with *L. major* parasites (4 out of 147; 2.7%). Similar infection rates have been reported by other researchers from various parts of the country (5, 20, 32, 34). Other species of the examined sand flies were not observed to be infected with *L. major* parasites. Furthermore, natural infection of *Ph. papatasi* and *Nesokia indica* with *L. major* has previously been reported from rural areas of Damghan District (13, 20). Based on the detection of *L. major* in *Ph. papatasi* collected from the studied area and the presence of ZCL human cases in this region, it may confirm the ZCL cycle in Damghan County.

Another species of the identified sand flies in this study, *Ph. sergenti*, were not shown to be infected with *Leishmania* parasites. *Phlebotomus sergenti* is the main vector of ACL in Iran and many other countries (35-39). Based on the presence of *Ph. sergenti* as the potential vector of ACL in Damghan City, further epidemiological study is recommended on human and animal reservoirs such as dogs.

Identification of *Ph. alexandri* was another important finding of this survey. Natural infection of *Ph. alexandri* with *L. infantum* as the causative agent of visceral leishmaniasis (VL) has already been reported from Iran (17, 40), and it has been introduced as vector of the disease in some other parts of the world (41-45). Moreover, the authors have recently reported the natural infection of *Nesokia indica* with *L. infantum* in the studied area (11). Therefore, beside of animal-sand fly-animal cycle of zoonotic visceral leishmaniais, animal-sand fly-human cycle of the disease can be expected in the studied region.

Phlebotomus caucasicus accounted for 6.2% of the surveyed sandflies. None of the species was found to be infected with *L. major* in this study. In a study, 3.3–20% of examined *Ph. caucasicus* collected from rodent barrows have been reported to be infected with *L. major* promastigotes in central part of Iran (4, 20, 40).

Most of the studied female sand flies (52.3 %) with digestive tract were checked for PCR. Of those, the largest number was belonging to *Ph. papatasi* and the largest number of infected sand flies (75%) was observed in the same specimens. In these sand flies, the parasites have passed blood digestion stages behind, and more importantly, they have been able to pass the peritrophic membrane (46).

In the current study, 75% of infected sand flies were trapped from rodent's burrows and outside places. Therefore, it is recommended that local people wear adequate dress and apply insect repellent to avoid bites outsides. In homes also the use of screens and bed nets impregnated with insecticide to prevent disease is recommended.

Conclusion

This study confirms the report of *Ph. papatasi* infection with *L. major* from Damghan City by other researchers (20). This finding could justify the reason for ZCL of people dwelling in the city with any history of travelling to the ZCL foci. Moreover, the existence of the other species of sand flies, including *Ph. sergenti* and *Ph. alexandri*, the vectors of *L. tropica* and *L. infantum* respectively, raises the possibility of future outbreaks of anthroponotic cutaneous leishmaniasis and visceral leishmaniasis in the study area. Therefore, further studies accompanied by designing and performing the vector control programs in the city are seriously recommended.

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Ethical considerations

Ethical approval for this study was obtained from the Ethics Committee at Semnan University of Medical Sciences, Iran (IR.SEMUMS. REC.1397.136).

Conflict of interest statement

Authors declare that there is no conflict of interest.

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