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ORIGINAL ARTICLE

Detection of *Leishmania major* DNA within wild caught *Phlebotomus papatasi* and species composition of sand flies in endemic focus of cutaneous leishmaniasis, in western Iran

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Abstract Cutaneous leishmaniasis is one of the most important public health problem in many developing countries. The present study was conducted to determine the vector(s), the parasite and the species composition of sand flies in the Dehloran County during May–November 2012. Sand flies were collected by sticky traps and mounted in Puri's medium for species identification. Polymerase chain reaction (PCR) techniques of kDNA, ITS1-rDNA, followed by restriction fragment length polymorphism (RFLP) were used for identification of DNA of *Leishmania* parasites in infected sand flies. A total of 82443 specimens comprising 15 species of sand flies (5 *Phlebotomus* and 10 *Sergentomyia*) were collected and identified. The species of *Phlebotomus papatasi* was dominant in outdoor and indoor resting places. Among the 280 specimens of female *P. papatasi* tested by PCR of kDNA, ITS1-rDNA genes of the parasite followed by RFLP, only 5 of them (1.8 %) were positive to *Leishmania major* parasites. This is the first molecular detection of *leishmania* infection of *P. papatasi* to *L. major* in this region. The results indicated that, *P. papatasi* was only species found infected by *L. major* and the principal vector of disease agent to human.

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Introduction

Leishmaniasis is still one of the world's most neglected diseases, affecting largely the poorest of the poor, mainly in developing countries. More than 350 million people are considered at risk of contracting leishmaniasis, and about 2 million new cases occur yearly (WHO 2010).There are four types of *leishmaniasis* in the world; Cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL). These diseases are widespread in the Old World and New World, with great epidemiological diversity. Theses diseases are transmitted to human by bite of female sand flies (*Phlebotomus* in the Old World and *Lutzomyia* in the New World). Twenty species of *leishmania* are important pathogens for human leishmaniasis. More than 700 species of sand flies are known worldwide, but ~70 species are serve as vectors of leishmaniases but only ≈30 species are important as proven vectors (Desjeux 2004; Rassi et al. 2006; Assimina et al. 2008).

There are several reports indicating occurrence of Cutaneaous Leishmaniasis due to *Leishmania major* (CLM) in Iran (Rassi et al. 2007, 2008, 2011a, b). CL is reported from 17 out of the 31 provinces in Iran (Afshar et al. 2012).

The annual incidence of CL has gradually increased in Iran and about 30,000 cases has been reported in 2011 (Unpublished data). This increasing trend is related to easily contact among human, sand fly and rodent contacts which is the result of the development of irrigation schemes and the spread of human populations into the habitats of the vectors and the rodents that act as reservoir hosts. About 80 % of reported cases of leishmaniasis in Iran are ZCL form (Yaghoobi-Ershadi 2012).

Dehloran county of Ilam province, west of Iran, is an important CLM focus where annually more than 300–400 cases of ZCL are reported (Ilam province health center, unpublished data). The main objectives of this study were to determine the sand fly species responsible for transmission of *L. major* to human and the parasite and the species composition of sand flies in the district.

Materials and methods

Study area

The study was carried out in 3 villages (Patake, Top Jlizy and Berahbijah) in Dehloran county (47°16E, 32°41N) at an altitude 215 m above sea level and 220 km far from Ilam city which has an average of 297.8 mm rainfall each year. Summers are hot that the temperature reaches to 53 °C. Winters are cool with average low temperatures of −0.6 °C. The relative humidity ranges are between 26 and 51 %. The total population of the study area was about 66,399 people in 2011 (Unpublished data). The major activities of population are agriculture and animal farming.

Sand flies collection

Sand flies were collected from indoors (bedroom, guestroom, toilet) and outdoors (rodent burrows, wall cracks) in a biweekly intervals using sticky traps. A total of 180 sticky traps were set up during the sand flies activity period (May–November) in 2011. All traps were installed at sunset and collected near sunrise. Collected sand fly specimens were washed once in 1 % detergent then twice in sterile distilled water. Each specimen was then dissected in a drop of fresh sterile normal saline by cutting off the head and abdominal terminalia with sterilized forceps and disposable needles. The rest of the body was stored in the sterile Eppendorf® micro tubes for DNA extraction. Specimens were mounted on glass slides using Puri's medium and identified using the identification keys for species within several subgenera (Absavaran et al. 2009; Theodor and Mesghali 1964; Lewis 1982).

DNA extraction

DNA was extracted by using the Bioneer® Genomic DNA Extraction Kit. Extraction was carried out by grinding of individual sand fly in a micro tube using glass pestle following the kit protocol and stored at 4 °C. Double distilled water was used as a negative control and DNA from *L. major* (MHOM/IR/75/ER) and *L. tropica* (MHOM/IR/03/Mash-878) provided by the Parasitology Department, School of Public Health, Tehran University of Medical Sciences (SPHTUMS) were used as positive controls (Oshaghi et al. 2009).

Sand flies DNA extraction

DNA of sand flies was extracted through *Kia Gene* Protocol. Individual female sand flies were homogenized with a sealed pasture pipette in 1.5 ml tubes. Then 100 µL lysis buffer [0.1 M Tris–HCl PH 7.5; 0.6 M NaCl; 0.1 EDTA] and 10 µL [0.8 M Tris–HCl PH 9.0: 0.27 M EDTA] were added and incubated at 65 °C for half an hour before 30 µL acetate potassium (8 mol) were added. After short centrifuge it was cooled for 45 min and transferred to new tube and added 350 µL cold pure ethanol and stored at −20 °C for 24 h. The tube was centrifuged at 13,000 rpm for 30 min then emptied the solution until dried and washed by 500 µL ethanol (75 °C) and the TE buffer was added.

Semi-Nested PCR for leishmania detection in sand flies

Semi-nested PCR was employed for detection of kinetoplast DNA (kDNA) of *L. major*in sand fly specimens. The primers were: LINR4 (Forward):5′-GGGGTTGGTGTA AAATAGGG-3′ (20 bp); LIN19 (Reverse): 5′-CAGAAC GCCCCTACCCG-3′ (17 bp), and LIN17 (reverse): 5′-TT TGAACGGGATTTCTG-3′ (17 bp). Positive samples were tested by PCR method against ITS1 gene using the primers (Forward): 5′-CTGGATCATTTTCCGATG-3′(18 bp) and (Reverse): 5′-TGATACCACTTATCGCACTT-3′ (20 bp). PCR amplification was followed by RFLP technique using *Hae*III enzymes for final species identification of the parasite. Reference strains *L. tropica* (MHOM/IR/89/ARD2) and *L. major* (MHOM/IR/54/LV39) were used as positive controls. All were obtained from the Medical Parasitology Laboratory, the School of Public Health, Tehran University of Medical Sciences, Iran.

Results and discussion

In total 82,443 sand flies comprising 15 species of sand flies (5 *Phlebotomus* and 10 *Sergentomyia*) were collected and identified. They included *Phlebotomus papatasi* (47.84 %), *P. caucasicus* (0.06 %), *P. alexandri* (1.1 %), *P. sergenti* (0.01 %), *P.* (*Adlerious*) sp. (0.8 %), *Sergentomyia sintoni* (16.2 %), *S. dentata* (16.9 %), *S. antennata* (0.8 %), *S. theodori* (0.1 %), *S. mervynae* (0.5 %), *S. irranica* (0.4 %), *S. squamipleuris* (0.2 %), *S. christophersi* (0.09 %), *S. clydei* (6.8 %) and *S. tiberiadis* (8.2 %). The species of *P. papatasi* was dominant specimen both outdoor and indoor places (Table 1). The activity of the species extended from May to November with a single peak in September (Table 2). Common specimens in resting places were *P. papatasi*, *P. Alexandri, S. sintoni*, *S. dentata*, *S.*

Table 1 Collected species of sand flies from outdoors and indoors, Dehloran county, Ilam province, 2011

Place species	Patake				Berahbijah				Top Jlizy				Total
	Indoors		Outdoors		Indoors		Outdoors		Indoors		Outdoors		
	M	F	M	F	M	F	M	F	M	F	M	F	
P. papatasi	8,516	7,761	4,854	4,959	1,512	875	1,459	1,617	2,547	1,854	2,315	1,176	39,445
P. cucausicus	3	–	20	–	–	–	5	–	3	–	20	–	51
P. alexandri	27	26	478	147	14	19	65	27	11	13	37	11	875
P. sergenti	–	6	–	5	–	–	–	–	–	–	–	–	11
P. (*adlerious*) sp.	–	60	–	74	–	174	–	251	–	97	–	35	691
S. sintoni	357	299	2,561	3,326	115	161	2,451	1,809	25	61	1,415	812	13,392
S. dentata	381	315	2,642	3,668	129	182	2,059	1,509	55	82	2,015	857	13,894
S. antennata	27	11	165	101	21	7	81	45	21	17	35	135	666
S. theodori	4	6	45	6	–	–	17	6	–	4	6	7	101
S. mervynae	6	7	53	112	–	–	51	106	–	–	24	41	400
S. irannica	12	–	177	–	7	–	58	–	6	–	74	–	334
S. squamipleuris	–	15	–	83	–	6	–	23	–	–	–	16	143
S. christophersi	–	–	55	–	–	–	–	–	–	–	19	–	74
S. clydei	–	–	1,225	1,233	–	–	875	911	–	–	711	613	5,568
S. tiberiadis	–	–	2,181	1,159	–	–	1,007	998	–	–	402	1,051	6,798
Total	9,333	8,506	14,456	14,873	1,798	1,424	8,128	7,302	2,668	2,128	7,073	4,754	82,443

M = Male F = Female

Table 2 Collected species of sand flies from, Dehloran county, Ilam province due to month of collection, 2011

Month species	May	June	July	August	September	October	November	Total
P. papatasi	52	1,544	2,954	6,798	21,344	5,455	1,298	39,445
P. cucausicus	–	–	12	24	15	–	–	51
P. alexandri	–	21	114	232	387	87	34	875
P. sergenti	–	–	–	4	7	–	–	11
P. adlerious	–	–	87	94	242	145	123	691
S. sintoni	62	1,223	2,432	2,655	5,434	1,243	343	13,392
S. dentata	121	1,343	2,465	3,487	4,623	1,432	423	13,894
S. antennata	37	59	39	203	223	73	32	666
S. theodori	–	–	26	31	44	–	–	101
S. mervynae	–	17	32	56	204	43	48	400
S. irannica	–	–	43	67	137	87	–	334
S. squamipleuris	–	–	27	49	67	–	–	143
S. christophersi	–	8	7	7	28	20	4	74
S. clydei	45	234	657	1,245	2,279	876	232	5,568
S. tiberiadis	117	277	768	1,435	2,786	1,089	326	6,798
Total	434	4,726	9,663	16,387	37,820	10,550	2,863	82,443

clydei and *S. tiberiadis*. The maximum and minimum sex ratio was calculated as 2.7 and 0.5 for *P. alexandri* and *S. mervynae* respectively (Table 3).

A total of 280 females of *P. papatasi* were surveyed to find *Leishmania* parasites. Only 5 (1.8 %) out of examined specimens of this species were positive for *L. major* using the semi- nested PCR of kinetoplast DNA. The visualized obtained bands in the infected specimens were similar to the standard strain of *L. major,* which was equal to 650 bp (Fig. 1). All of the infected sand flies had been collected from indoor places. Their abdominal stages were either gravid or empty indicating there was enough time for the parasites to develop and to transform to promastigote, the infective form. Further analyses showed that they were positive against ITS1 locus and produced a band of ~340 bp in gel electrophorese. Also, ITS1 PCR–RFLP analysis by HaeIII revealed the fragments of 220 and 140 bp for infected sand flies which are characteristic of *L. major.*

The diagnostic fragments are 200 and 60 bp for *L. tropica* (Figs. 2, 3).

Table 3 Sex ratio of collected sand flies, Dehloran county, Ilam province, 2011

Gender Species	Male	Female	Sex ratio
P. papatasi	21,203	18,242	1.2
P. cucausicus	51	–	–
P. alexandri	632	243	2.7
P. sergenti	–	11	–
P. adlerious	–	691	–
S. sintoni	6,924	6,468	1.1
S. dentata	7,281	6,613	1.1
S. antennata	350	316	1.1
S. theodori	72	29	2.5
S. mervynae	134	266	0.5
S. irannica	334	–	–
S. squamipleuris	–	143	–
S. christophersi	74	–	–
S. clydei	2,811	2,757	1.02
S. tiberiadis	3,590	3,208	1.1
Total	43,456	38,987	1.1

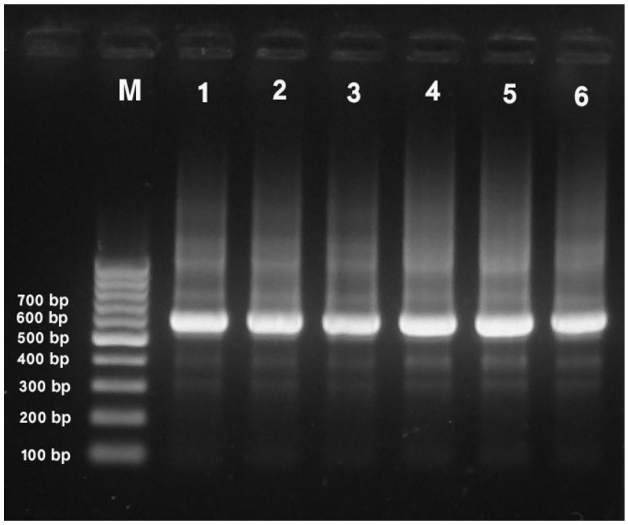

Fig. 1 Minicircle kDNA PCR amplification of *L. major* in *P. papatasi* using Semi-nested PCR. *M* molecular size marker, *1 Leishmania major* standard, *2–6* samples of infected *P. papatasi* to *L. major*

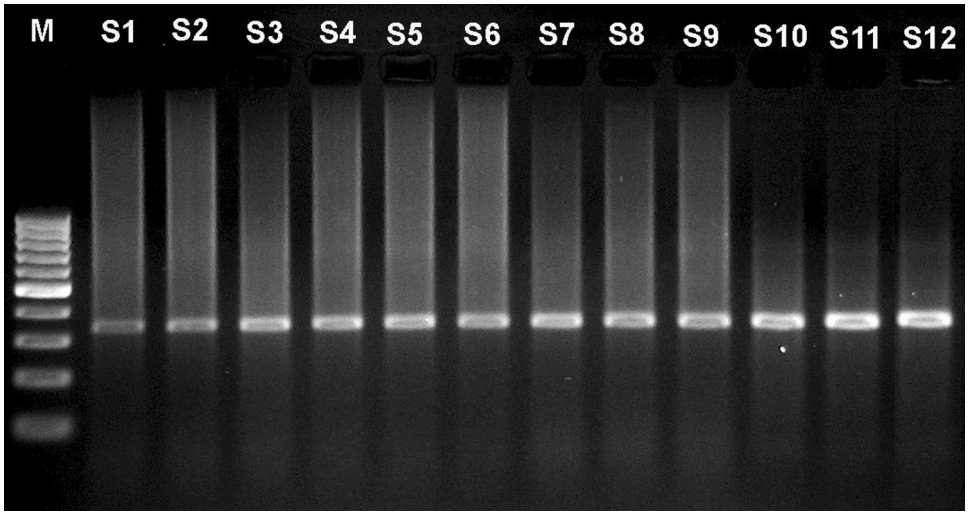

Fig. 2 ITS1 amplification of *L. major* in *P. papatasi* using nested PCR primers. *M* marker, *S1–S10* samples of infected *P. papatasi* to *L. major*, *S11* positive control of *L. major*, *S12* *L. tropica* as negative control

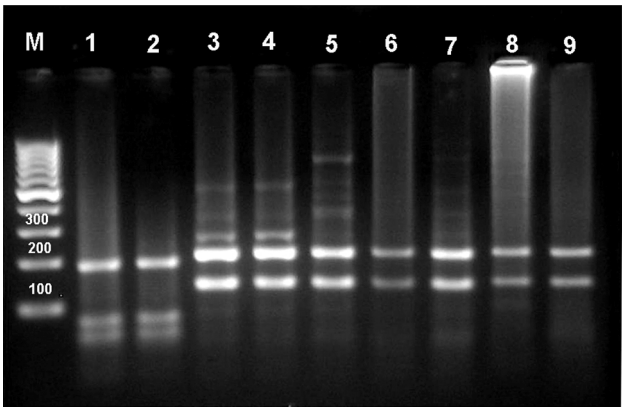

Fig. 3 PCR-RFLP analysis of ITS1 region for identification of *Leishmania* species using HaeIII *M* marker, *1–2* negative control of *L. tropica*, *3–4* positive control of *L. major*, *5–9* samples of infected *P. papatasi* to *L. major*

The ITS1 DNA sequences which were obtained from 5 positive infected *P. papatasi* specimens submitted to Genbank database with accession numbers: KC880112, KC880113 (rodent burrow specimens) and, KC880114, KC880115, KC880116 (indoors specimens). Comparison of these sequences with other available data, confirming it as *L. major*.

All specimens were identical (100 %) to several *L. major* sequences deposited in Genbank, including isolates from Brazil (Accession No. DQ300195), Iran (Accession No. AY260965), Kenya (Accession No. AJ300482). Also they were found to be 100 % similar to *Leishmania mexicana venezuelensis* from Mexico (Accession No. AF339752).

Epidemiology of leishmaniasis and ecology of sand flies are important factors to management and planning for disease control. Entomological survey with epidemiological data are very important for control planning against *leishmania* disease (Rassi et al. 2008). Anthropophily of sand flies and common infection of them with same *leishmania* parasite that found in man in the same places, indicate the capacity of sand fly as a vector (Killick-kendrick 1990). For more verification, Molecular techniques PCR have been used. This technique is highly sensitive to detection of *leishmania* parasites in sand flies and commonly used in Iran and other countries (Rassi et al. 2006, 2007, 2008, 2011a, b, 2012a, b; Mukherjee et al. 1997; Azizi et al. 2006; De Bruijn and Barker 1992). The fact that *P. papatasi* females were found indoors, with gravid or with empty abdomen, suggests a considerable vectorial capacity of this species. These findings showed the direct effects of population density and anthropophilic behavior of a sand fly species in vector borne diseases. The result of the current study revealed all of the important factors present for establishment of the disease in the region. These include human activities close to rodent burrows, the presence of high density of *P. papatasi* in the rodent burrows and indoors, and proximity of human habitat to rodent colonies, which have led to emergence of a new focus of *L. major* in the region. Furthermore 100 % similarity of *L. major* isolated in collected sand flies (*P. papatasi*), from indoors and rodent burrows implies, this species circulate the parasite (*L. major*) between rodents as a reservoirs of disease and human. As a final conclusion it can be concluded that *L. major* is the cause of and *P. papatasi* is the primary vector of CL in Dehloran county west of Iran. It is noticeable that *P. papatasi* is the main proven vector of ZCL in Iran (Rassi et al. 2011a).

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