First Detection of *Leishmania infantum* in *Phlebotomus* (*Larroussius*) *major* (Diptera: Psychodidae) from Iran

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ABSTRACT Ghir-Karzin district is one of the most important endemic foci of visceral leishmaniaisis (VL) in Fars province of southern Iran. To identify the vector(s) of *Leishmania* in this focus, a total of 2,539 sand flies were collected during June to September 2005. Eleven species (six *Phlebotomus* and five *Sergentomyia*) were identified, of which *Phlebotomus papatasi* Scopoli and *Phlebotomus major* Annandale were the first (37.4%) and third (11.2%) most common species, respectively. Natural leptomonad infections were observed in two *P. papatasi* (4.25%) and three *P. major* (6.65%) specimens on dissection and microscopic examination. Using a *Leishmania* genus–specific standard polymerase chain reaction (PCR; primers RV1-RV2) and a species-specific nested-PCR (primers LINR4, LIN17, and LIN 19), *Leishmania infantum* and *Leishmania major* kinetoplast minicircle DNA was detected in 6 of 72 *P. major* (8.3%) and 4 of 65 *P. papatasi* (6.1%), respectively. This is the first detection of *L. infantum* in *P. major*, implicating this sand fly as a probable vector of VL in Iran.

KEY WORDS visceral leishmaniasis, *Leishmania infantum*, *Phlebotomus major*, nested polymerase chain reaction, Iran

The Leishmaniases are a group of diseases caused by infection with protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). They are distributed worldwide and affect at least 12 million people annually. More than 20 species of *Leishmania* have been described as causative agents of human leishmaniasis, and specific clinical features are largely associated with each *Leishmania* species (Kato et al. 2005). Visceral leishmaniasis (VL), which is commonly caused by *Leishmania infantum* in the Mediterranean basin, the Middle East, and Latin America, affects approximately half a million new patients each year (Lachaud et al. 2002). Because of systemic parasite dissemination, VL is the most severe form of leishmaniasis, which is nearly always fatal if left untreated (Shyam and Rai 2002). Phlebotomine sand flies (Diptera: Psychodidae) are the sole vectors of *Leishmania*, and species of the genus *Phlebotomus* are the only known vectors in the Old World (Alexander and Maroli 2003). Although >700 sand fly species have been described, only a few (≈50) have been shown to be able to support the development of *Leishmania* species and thus are vectors of disease (Alexander 2000).

Although VL is seen sporadically throughout Iran, there are three important endemic foci: Ardebil and East-Azerbaijan in the northwest and Fars province in the south. Although wild and domestic carnivores are commonly considered the main reservoirs, rodents have also been reported as reservoirs in the Meshkinshahr district in northwestern Iran (Edrissian et al. 1999, Mohebali et al. 2005). Two sand fly species, *Phlebotomus* (*Paraphlebotomus*) *alexandri* Sinton and *Phlebotomus* (*Larroussius*) *kandelakii* Shschurenkova, have been reported as the proven or probable vectors of *L. infantum* in Iran (Azizi et al. 2006, Rassi et al. 2005). Three other species, *Phlebotomus* (*Larroussius*) *keshishiani* Shschurenkova, *Phlebotomus* (*Larroussius*) *perfiliewi* Parrot, and *Phlebotomus* (*Larroussius*) *major* Annandale, have been found naturally infected with promastigotes and are suspected vectors of VL in the country (Sahabi et al. 1992, Seyyedi Rashti et al. 1995, Rassi 1997).

Ghir-Karzin district is one of the most important endemic foci of VL in Fars province, with >10 cases of disease (all aged <10 yr) annually. Previously in this district, two sand fly species (*P. keshishiani* and *P. major*) were reported to be naturally infected with promastigotes on dissection and microscopic examination.

The aim of this study was to identify the vector(s) of VL in this focus using dissection and microscopic examination, as well as molecular techniques such as nested and standard polymerase chain reaction (PCR).

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Materials and Methods

Study Area. The study was carried out in 2005 in Ghir-Karzin district, Fars province, in southern Iran. This district is situated in a hilly area, south of the Zagross mountain range (53°10′ E, 28°25′ N) at an altitude of 780 m. Ghir is a small town in this district with >25,000 inhabitants. The average maximum and minimum temperatures in the summer are 42 and 19°C and in winter are 21 and 3°C, respectively. The relative humidity ranges from 27 to 85%, and the annual precipitation is ≈204 mm.

Sand Fly Collection. Sand flies were caught at three stations, Shahrak-e-emam, Sekkeh-Ravan, and Ghir suburbs, where cases of VL had been reported, using CDC miniature light traps, sticky traps, and mouth aspirators. Collection of sand flies was performed monthly for 3–5 consecutive d from June to September 2005. Sixty sticky traps and three light traps were used at each station per night. The male flies were stored in 70% ethanol for subsequent mounting and species identification. The females were selected for dissection and DNA extraction.

Dissection and Identification of Sand Flies. Sand flies were washed in 1% detergent solution for 2 min and dissected in a drop of normal saline (pH 7.2) and examined for promastigotes. The head and last abdominal segments were mounted on a microscope slide in a drop of Puri medium (Smart 1965), so that each fly could be identified to species, according to the keys provided by Lewis (1982). The remaining portion of each unfed, parous female of the more common *Phlebotomus* species (*P. major*, *Phlebotomus papatasi* Scopoli, *P. alexandri*, and *Phlebotomus sergenti* Parrot) was used for DNA extraction and PCR. Parity was determined based on the presence or absence of yellowish pigments in the accessory glands.

DNA Extraction. Total DNA was extracted from each sand fly body as described elsewhere (Azizi et al. 2006). Briefly, each body was homogenized with 200 μl lysis buffer (50 mM Tris-HCl [pH 7.6], 1 mM EDTA, and 1% Tween 20) and 12 μl of a proteinase K solution (containing 19 μl of the enzyme/ml) in a 1.5-ml microcentrifuge tube. The process continued by adding 300 μl of a phenol:chloroform:isoamyl alcohol mixture (25:24:1 by volume). The mixtures were centrifuged, and the DNA was precipitated with 400 μl cold, pure, ethanol, resuspended in 50 μl double-distilled water (DDW), and stored at −20°C until DNA was amplified by PCR.

For promastigote DNA extraction from specimens found positive by dissection and microscopic examination, the microscopic slides were washed three times with lysis buffer, and the lysates were transferred to microtubes and processed as described above. All experiments were performed in the Medical Parasitology Laboratory, Faculty of Medicine, Shiraz University of Medical Sciences.

***Leishmania* Reference Strains.** Reference strains of *L. infantum* (MCAN/IR/96/Lon 46), *L. major* (MHOM/IR/54/LV 39), and *L. tropica* (MHOM/IR/89/ARD 2) were used as standards. All were obtained from the Medical Parasitology Laboratory, the School of Public Health and Institute of Health Research, Tehran University of Medical Sciences.

Amplification of Kinetoplast Minicircle DNA from Sand Flies. An assay based on the seminested PCR and a slight modification of the protocol described by Aransay was used to amplify the variable area of the minicircle kDNA of any *Leishmania* present in the sand fly bodies (Aransay et al. 2000). This method was changed to a two-step nested PCR, each step of which was carried out in a separate tube, with the product of the first step being diluted with DDW (4:1) and used as a template for the second step (nested).

The forward primer LIN R4 (5′-GGGGTTGGTGTAAAATAGGG-3′) was used for both steps, and primers LIN 17 (5′-TTTGAACGGGATTTCTG-3′) and LIN 19 (5′-CAGAACGCCCCTACCCG-3′) were used as reverse primers in the first and second steps, respectively. These primers were designed within the conserved area of the minicircle and contained the conserved sequence blocks 3, 2, and 1, respectively (Aransay et al. 2000).

The first step (amplification reaction) was carried out in a total of 25 μl containing 250 μM of each dNTPs, 1.5 mM $MgCl_2$, 1U *Taq* polymerase (Cinagene, Tehran, Iran), 1 μM primer LIN R4, 1 μM primer LIN 17, and 5 μl of DNA extract in 1× PCR buffer (Boehringer Mannheim, Mannheim, Germany). The mixture was incubated in a CG1–96 thermocycler (Corbett Research, Sydney, Australia) set to give 5 min at 94°C, followed by 30 cycles, each cycle consisting of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C, and a final extension at 72°C for 10 min and held at 4°C.

The second step (nested) was carried out in a separate tube containing a 20-μl reaction mixture of 1× *Taq* polymerase buffer, $MgCl_2$, dNTPs, and *Taq* polymerase as described for the first step, plus 1 μM primer LIN R4, 1 μM primer LIN 19, and 2 μl of the amplification reaction mixture (diluted 4:1 with DDW). The PCR profile was the same as in the first step except it was run for 33 cycles.

The second method was a standard PCR based on the protocol described by Le Fichoux et al. (1999) (with some modification) using RV1 (forward) (5′-CTTTTCTGGTCCCGCGGGTAGG-3′) and RV2 (reverse) (5′-CCACCTGGCCTATTTTACACCA-3′) primers to amplify a 145-bp sequence of LT1 in the conserved region of kinetoplast DNA minicircles (Le Fichoux et al. 1999). PCR was carried out using 5 μl of DNA solution in a final volume of a 25-μl reaction mixture containing 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 0.1 U of *Taq*DNA polymerase (Cinagene), 1 μM of each primer, and 5 μl of DNA extract in 1× PCR buffer (Boehringer Mannheim). Each reaction mixture was overlaid with mineral oil before being transferred to a CG1–96 thermocycler (Corbett Research). After an initial denaturation (4 min at 94°C), 40 cycles (denaturation, 30 s at 94°C; annealing, 30 s at 59°C; polymerization, 30 s at 72°C) were carried out, and PCR was terminated by a final extension at 72°C for 10 min and held at 4°C.

Table 1. Species diversity and relative abundance of sand flies collected in Ghir-Karzin district in 2005 and their *Leishmania* infection rates by microscopic examination and PCR

Species	No. collected				No. and % parous females examined for *Leishmania* DNA (PCR)		No. and % females dissected and examined for promastigotes	
	Males	Females	Total	% total	Number examined	Number infected	Number examined	Number infected
P. (Phlebotomus) papatasi	563	387	950	37.4	65	4 (6.15)	47	2 (4.26)
P. (Larroussius) major	105	179	284	11.2	72	6 (8.33)	45	3 (6.65)
P. (Paraphlebotomus) alexandri	75	93	168	6.6	32	0 (0)	39	0 (0)
P. (Paraphlebotomus) sergenti	90	53	143	5.6	12	0 (0)	10	0 (0)
P. (Phlebotomus) bergeroti	45	39	84	3.3	—	—	—	—
P. (Phlebotomus) salehi	15	19	34	1.3	—	—	—	—
S. (Sergentomyia) dentata	210	320	530	20.9	—	—	—	—
S. (Sergentomyia) sintoni	31	92	123	4.8	—	—	—	—
S. (Sintonius) clydei	19	87	106	4.2	—	—	—	—
S. (Parrotomyia) baghdadis	17	51	68	2.7	—	—	—	—
S. (Sergentomyia) theodori	17	32	49	1.9	—	—	—	—
Total	1,187	1,352	2,539	100	181	10 (5.52)	141	5 (3.54)

Negative controls (DNA extracted from a male sand fly and an aliquot of distilled water) were included in each PCR run to detect contamination that could lead to false-positive results; all were found to be negative.

A 5-μl sample of each PCR product was subjected to electrophoresis in a 1.5% agarose gel. The bands were stained with ethidium bromide and visualized under UV trans-illumination.

Parasites were identified by comparing the size of the band produced from a test sample with those produced from the reference strains of *L. infantum*, *L. major*, and *L. tropica*. For example a band of 720 bp (in the nested PCR method) indicated that the parasite was *L. infantum*.

Results

A total of 2,539 sand flies (1,187 males and 1,352 females) were collected from three different locations, among which 11 Phlebotomine species were identified, including six species of Phlebotomus and five species of Sergentomyia (Table 1). *P. (Phlebotomus) papatasi* Scopoli and *P. (Larroussius) major* Annandale were the first and third most prevalent species in the collection, representing 37.4 and 11.2% of the total sand flies caught, respectively. Altogether, 47 *P. papatasi*, 45 *P. major*, 39 *Phlebotomus alexandri* Sinton, and 10 *Phlebotomus sergenti* Parrot were dissected and examined microscopically for promastigotes. The results of the dissection are presented in Table 1. Promastigotes were observed only in two specimens of *P. papatasi* and three specimens of *P. major* (Fig. 1).

The promastigote-positive *P. papatasi* specimens were collected outdoors from rodent burrows on sticky traps, but all of the infected specimens of *P. major* were caught in and around the VL patients' dwellings using sticky and CDC light traps (five and one specimens, respectively).

Altogether, 65 *P. papatasi*, 72 *P. major*, 32 *P. alexandri*, and 12 *P. sergenti* (including slides of dissected specimens) were checked for *Leishmania* DNA using a genus-specific PCR (RV1-RV2 primers) and a species-specific nested PCR (primers LIN R4, LIN 17, and LIN 19). Four specimens of *P. papatasi* and six of *P. major* were found to be positive for *Leishmania* DNA using standard PCR. All of them had a 145-bp band indicative of the LT1 fragment in the conserved region of *Leishmania* kinetoplast DNA (kDNA) minicircles (Fig. 2).

The nested PCR method could identify these parasites as *Leishmania major* and *L. infantum* with primers LIN R4, LIN 17, and LIN 19. The size of all amplified products from *P. papatasi* specimens was ≈560 bp, which was equal to the band size of the *L. major* standard strain.

The visible bands obtained from *P. major*–infected specimens coincided with those of the *L. infantum* standard strain, equal to 720 bp. The obtained bands of standard strains of *L. major*, *L. infantum*, and *L. tropica* were 560, 720, and 760 bp, respectively (Fig. 3).

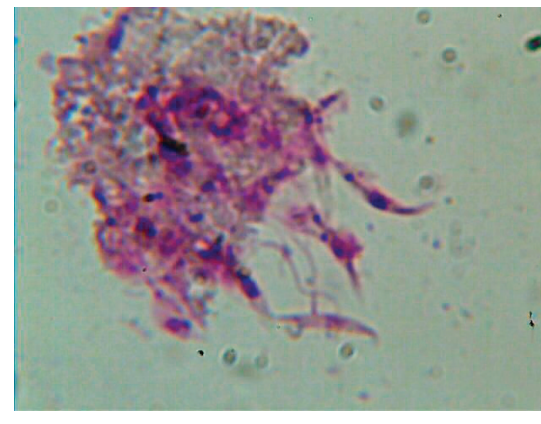

Fig. 1. Geimsa-stained promastigotes of *L. infantum* from a naturally infected, wild-caught, *P. major* female sand fly collected in Ghir-Karzin district, Fars province, 2005.

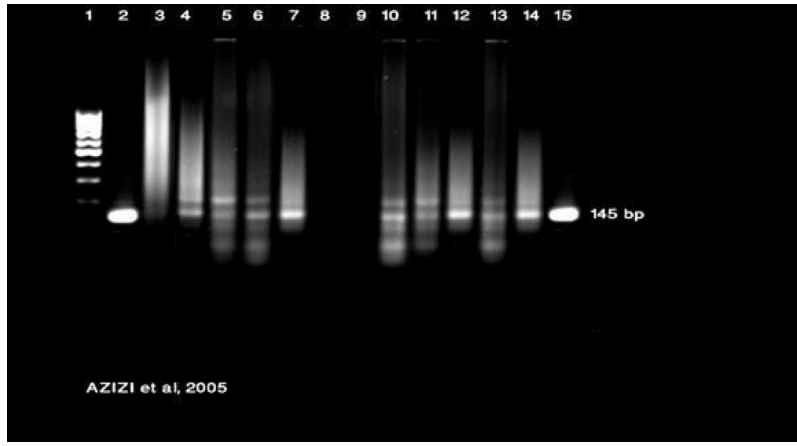

Fig. 2. The results of the standard PCR-based amplification of kinetoplast DNA. The bands correspond to molecular weight marker (lane 1), reference strains of *L. infantum* and *L. major* (lanes 2 and 15), samples of *P. papatasi* (lanes 3–7), samples of *P. major* (lanes 10–14), a male sand fly as control (lane 8), and blank (lane 9).

Discussion

Diagnosis of vector species and proper knowledge of their biology are absolutely essential for designing effective control programs for vector-borne diseases. Finding naturally infected, wild-caught specimens is essential evidence in incrimination of a sand fly species as a vector. However, only parasite isolation and/or identification can confirm the vector. There are two classical procedures used for detection of *Leishmania* in suspected vectors: microscopic dissection, which is not definitive because most flagellate protozoa are morphologically indistinguishable, and culture of isolated parasites, which often does not give results because of failure to grow or contamination (Aransay et al. 2000). Dissection of sand flies to examine them microscopically for promastigote infections is time-consuming and requires highly skilled microscopists.

Molecular methods using different PCR techniques are powerful and efficient tools for detection and identification of *Leishmania* DNA within sand flies. The PCR technique is a highly sensitive and powerful

Fig. 3. The results of the nested PCR-based amplification of kinetoplast DNA. The bands correspond to molecular weight markers (lanes 1 and 8), reference strains of *L. tropica* (lane 2), *L. major* (lane 3), *L. infantum* (lane 4), two of the infected *P. major* specimens (lanes 6 and 7), and a male sand fly as control (lane 5). The bands are shown on a 1.5% agarose gel stained with ethidium bromide.

tool, which has been used by others for detecting *Leishmania* DNA in naturally infected sand flies (De Bruijn and Barker 1992, Mukherjee et al. 1997, Rodriguez et al. 1999).

One of the most suitable targets for PCR is minicircle kinetoplast DNA because of the high number of copies per parasite cell (10,000), and a well-known sequence of the variable region, which has high diversity in different species, making them easily distinguishable (Aransay et al. 2000).

In this study, infection of *P. major* by *L. infantum* was confirmed using both microscopic and molecular methods. *P. major* has a wide distribution from Morocco to southeast China. It is a main vector of VL in Greece and is also considered to be a vector of VL in other countries in the Mediterranean basin (Hoogstral and Heyneman 1969, Leger et al. 1979). This species has been incriminated as a possible vector of *L. infantum* in the western Black Sea region of Turkey (Daldal et al. 1998).

In Iran, *P. major* was first reported from northern parts and later from other parts of the country, in all areas where human cases of VL have been reported. Several workers have suspected *P. major* as the main vector of VL in Iran, on the basis of epidemiological evidence (Javadian and Nadim 1975, Nadim et al. 1978).

Sahabi et al. (1992) found 5 of 150 dissected *P. major* (3.3%) naturally infected with promastigotes in Ghir-Karzin district (our study area), further implicating this species as a probable vector of VL in Iran (Sahabi et al. 1992).

The standard PCR-based assay used in this study was successfully used by Le Fichoux et al. (1999) to amplify *Leishmania*-kinetoplast minicircle DNA from blood samples of asymptomatic blood donors who lived in an endemic area of VL in southern France. This method amplified the LT1 (145 bp) sequence in the conserved region of *Leishmania*-kDNA minicircles.

Aransay et al. (2000) used the primers LINR4, LIN 17, and LIN 19 in a seminested PCR for amplification of the variable region of *Leishmania*-kDNA minicircles in wild populations of *P. alexandri*, *P. tobbi*, *P. neglectus*, *P. simici*, and *P. papatasi* in Greece. Their assay was carried out in a single tube, whereas we used two separate tubes for a truly nested PCR.

The standard PCR assay was *Leishmania* genus specific and amplified a 145-bp band in all of our *Leishmania* reference strains (Fig. 2), whereas the nested PCR amplified the different sized bands for these species and could clearly differentiate among them (The resulting bands were 560, 720, and 760 bp for *L. major*, *L. infantum*, and *L. tropica*, respectively; see Fig. 3).

The two PCR methods used in this study have the same sensitivity and efficiency for detecting *Leishmania* infections in naturally infected sand flies and both *Leishmania*-kDNA in the same infected sand flies (four specimens of *P. papatasi* and six specimens of *P. major*).

All *P. major* and *P. papatasi* specimens found infected in this study were parous, indicating longevity sufficient to complete the parasite cycle in their bodies. Furthermore, the infected flies showed no sign of a blood meal, and therefore, when caught, probably harbored promastigotes that had developed from amastigotes ingested, within a blood meal, at least several days earlier (Cihakova and Volf 1997).

Based on (1) observations of promastigotes in naturally infected, wild caught, specimens of *P.* (*Larroussius*) *maor* (which were collected from VL patients' homes); (2) identification of the parasites as *L. infantum* by species-specific nested PCR; (3) high abundance of this man-biting sand fly species in this endemic focus (11.2%); (4) absence the other suspected vectors of *Leishmania;* and (5) failure to find other *Phlebotomus* species naturally infected with *Leishmania* parasites, we conclude that *P. major* should be considered as the primary vector of VL in this endemic focus in Fars province, southern Iran.

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