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## *Leishmania infantum* DNA detection in *Phlebotomus tobbi* in a new northern focus of visceral leishmaniasis in Iran

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## PEER REVIEW

## Peer reviewer

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## Comments

The authors could identify a sand fly vector of VL in the new focus of disease, northern Iran, using microscopic examination, as well as molecular techniques, including PCR of kDNA, ITS1-rDNA, followed by RFLP. The results may help the control of *P. tobbi* as a target vector in the area. Details on Page 113

## ABSTRACT

**Objective:** To identify the vector(s), the parasite and the species composition of sand flies in the district during May–October 2012.

**Methods:** For reaching our objectives we used polymerase chain reaction of kDNA, ITS1-rDNA, followed by restriction fragment length polymorphism.

**Results:** Two species of *Phlebotomus sergenti* and *Phlebotomus tobbi* were the most prevalent among 8 species identified comprising 51.1% and 32.9% respectively. Among the 160 specimens of female sand flies tested by polymerase chain reaction of kDNA, ITS1-rDNA, followed by restriction fragment length polymorphisms, only 1 out of 80 *Phlebotomus tobbi* (1.25%) were positive to *Leishmania infantum* parasites.

**Conclusions:** Our finding showed that *Phlebotomus tobbi* may play as a vector to circulate the parasite of *Leishmania infantum* among reservoir(s) and human.

## KEYWORDS

*Leishmania infantum*, Vector(s), Alborz, Iran

### 1. Introduction

The human leishmaniasis are the most diverse and complex of all vector-borne diseases in their ecology and epidemiology. *Leishmania infantum* (*L. infantum*), belonging to the *Leishmania donovani* (*L. donovani*) complex, is the causative agent of human visceral leishmaniasis (VL), cutaneous and canine visceral leishmaniasis in all Mediterranean countries. The disease is zoonotic and transmitted to humans from its reservoir hosts, mainly dogs by the bite of sand flies of genus

*Phlebotomus*[1,2]. Because of systemic parasite dissemination, VL is the most severe form of leishmaniasis, which is nearly always fatal if left untreated[3].

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[4]. 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[2].

There are several reports on sporadically occurrences of

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zoonotic VL in Iran, however, the disease is endemic in six provinces including Ardebil, East Azerbaijan (Northwestern), Fars, Bushehr, Kerman (South) and Khorassan Shomali (North eastern) of Iran[5–9].

Three species of sand flies including *Phlebotomus kandelakii*, *Phlebotomus perfiliewi transcaasicus* and *Phlebotomus tobbi* (*P. tobbi*) have been reported to be vectors of the disease in north western and north eastern of Iran[6,9,10–12]. The species of *Paraphlebotomus alexandri* (Sinton) and *Phlebotomus (Larroussius) major* (Annandale) have been found naturally infected with *L. infantum* and are the VL vectors in the south part of the country.

*Phlebotomus (Larroussius) keshishiani* (Shchurenkova) and *Phlebotomus (Paraphlebotomus) caucasicus* have been reported infected with promastigotes in southern Islamic Republic of Iran but no parasite identification was carried out[13,14].

The important aims of this study was to identify the vector(s) of zoonotic visceral leishmaniasis and species composition of sand flies in the new focus of disease, northern Iran, using dissection and microscopic examination, as well as molecular techniques.

## 2. Materials and methods

### 2.1. Study area

This study was carried out from early May to late October 2012 in 4 villages, Kordan, Aghasht, Arab–Abad and Hiv in Savodjbolagh county, Alborz province, north of Iran (Figure 1). The capital of the county is Hashtgerd. At the 2006 census, the county's population (including those portions later split off to form Taleqan county) was 215086, in 57497 families. The county is subdivided into three districts: the Central district, Chaharbagh district, and Chendar district. The county has five cities: Hashtgerd, Chaharbagh, Golsar, Kuhsar, and Shahr–e Jadid–e Hashtgerd. The mean annual precipitation is 290 mm with 53% relative humidity, and the mean annual temperature is 17.2 °C. The maximum and minimum average monthly temperatures were 28.3 °C and 7.4 °C respectively.

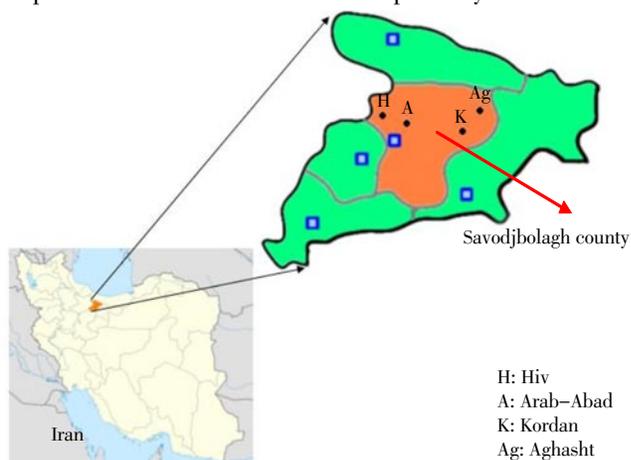


Figure 1. Map of the study area.

### 2.2. Sand flies collection and identification

Sand flies were collected using sticky traps (castor oil–coated white papers 15 cm×20 cm) biweekly from indoors (bedrooms, guest room, toilet and stable) and outdoors (rodent burrow). Traps (60 papers per village) were set at dusk and sand flies

were collected at dawn. Sample collection began in early May and continued until late October 2012.

The 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 micro tubes for DNA extraction. Specimens were mounted on glass slides using Puri's medium and identified using the identification keys for species within *Larroussius* group and for species of other groups and subgenera[15–17].

### 2.3. DNA extraction

DNA was extracted by using the Bioneer Genomic DNA Extraction Kit. Extraction was carried out by grinding of individual sand flies 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 *Leishmania major* (*L. major*) MHOM/IR/75/ER, *Leishmania tropica* (*L. tropica*) MHOM/IR/03/Mash–878; *L. infantum* MHOM/FR/87/LEM1098 provided by the Parasitology Department, School of Public Health, Tehran University of Medical Sciences were used as positive controls. DNA from *L. donovani* IPER/IR/2007/HS10 (isolated previously from sand flies) was used as positive control[18].

### 2.4. Detection and identification of *Leishmania* species

Initial screening of sand flies was performed by nested–PCR amplification of kinetoplast DNA (kDNA) using the primers (Table 1) and protocol described by Noyes *et al.*, 1998[19]. This method is highly sensitive and is recommended for initial screening. Amplification was carried out in two steps; both in the same tube. This PCR protocol is able to identify *Leishmania* parasites by producing a 680 bp for *L. infantum/L. donovani*, 560 bp for *L. major*, and a 750 bp for *L. tropica*. The cycling conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds, and 72 °C for 90 seconds. One micro liter of a 9:1 dilution in water of the first–round product was used as template DNA for the second round in a total volume of 30 mL under the same conditions as those for the first round, except with primers LIR and 13Z. Due to presence of many DNA polymorphisms in kDNA of each *Leishmania* species, sequencing of kDNA is problematic. Therefore, further identification of the *Leishmania* parasites was done using the ITS1–PCR followed by *Hae* III digestion of the resulting amplicons[19,20]. A set of primers (Table 1) LITSR and L5.8S was used to amplify 340 bp of rDNA including parts of 3' end of the 18S rDNA gene, complete ITS1, and part of 5' end of the 5.8S rDNA gene. PCR products (15 µL) were digested with *Hae* III without prior purification using conditions recommended by the supplier (Cinagen, Iran).

Table 1

List and specification of primers used in this study.

PCR step	Name	Sequences (5'–3')
First	CSB2XF	CGAGTA GCAGAAACTCCCGTTCA
	CSB1XR	ATTTTCGCGATTTCGCAGAACC
Second	13Z	ACTGGGGGTTGCTGTAATAATAG
	LIR	TCCGAGAACGCCCT
One	LITSR	CTGGATCATTTCCGATG
	L5.8S	TGATACCACTTATCGCACT

Also, the ITS1 PCR products (340 bp) of the samples that

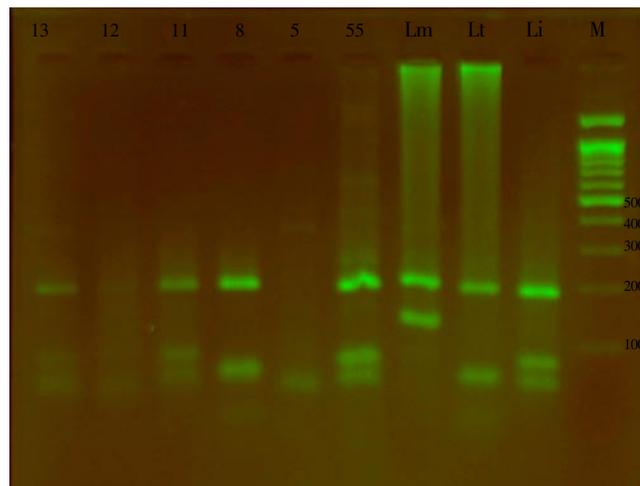
demonstrated *L. infantum* profile were sequenced (SeqLab, Göttingen, Germany), employing the same primers used for the PCR. The sequences obtained were processed and aligned, using the multiple alignment program Clustal X[21]. Homologies with the available sequence data in GenBank was checked by using basic local alignment search tool (BLAST) analysis software (<http://www.ncbi.nlm.nih.gov/BLAST>). A PCR protocol of 30 cycles of denaturation 30 seconds at 94 °C, annealing 1 min at 62 °C and elongation 1 min at 72 °C, followed by a final elongation of 10 min at 72 °C was used. All PCR products were analyzed by 1–1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light. Standard DNA fragments (100 bp ladder, Fermentas) were used to permit sizing. PCR products (15 µL) of ITS1 were digested by *Hae* III without prior purification using conditions recommended by the supplier (Cinagen, Iran). The restriction fragments were subjected to electrophoresis in 2% agarose and visualized under ultraviolet light after staining for 15 min in ethidium bromide (0.5 µg/mL). *Hae* III digestion of ITS1 PCR reveals two fragments of 220 and 140 bp for *L. major*, fragments of 200, 80 and 60 bp for *L. infantum/donovani*, and two fragments of 200 and 60 bp for *L. tropica*.

### 3. Results

Altogether 7930 sticky traps were installed and 1034 specimens comprising 8 species of sand flies were collected and identified. They included *Phlebotomus sergenti* (51.06%), *P. tobbi* (32.97%) *Phlebotomus major* (1.74%), *Phlebotomus papatasi* (2.14%), *Phlebotomus kandelakii* (1.26%), *Phlebotomus caucasicus* (6.96%), *Phlebotomus perfiliewi transcaucasicus* (3.19%) and *Paraphlebotomus alexandri* (0.68%). *Phlebotomus sergenti* and *P. tobbi* were the dominant *Phlebotomus* species respectively (Table 2). The activity of sand flies started in May and ended in October with one peak in early August. Sex ratio (male to female) of the sand flies captured in the region were 164/100 in indoor and outdoors.

Overall a total of 160 dominant female sand flies including *P. tobbi* (50%) and *Phlebotomus sergenti* (50%) were tested for *Leishmania* parasite genome. Only 1 out of 80 specimens of *P. tobbi* (1.25%) were positive for *L. infantum/donovani* using the nested PCR against kinetoplast DNA. This was observed in the kDNA nested-PCR amplification assays where a 680 bp PCR band was produced. This length of PCR in the system is assigned to *L. infantum/L. donovani*. Examination of the

one infected specimens showed that its abdomen was empty. Further analysis showed that it was positive against ITS1 locus and produced a band of approximately 340 bp. RFLP analysis by *Hae* III revealed the fragments of 200, 80 and 60 bp for *L. infantum* which are characteristic of *L. infantum/donovani*. The diagnostic fragments were 220 and 140 bp for *L. major* and 2 fragments of 200 and 60 bp are for *L. tropica* (Figure 2).



**Figure 2.** ITS1-rDNA PCR-RFLP analysis of selected strains of *Leishmania* species using *Hae* III restriction enzyme.

M: 100 bp ladder (Fermentas); Lm: *L. major* (MHOM/IR/75/ER: serial code of the standard *L. major* species at the reference laboratory of the Parasitology Department, Tehran University of Medical Sciences); Lt, *L. tropica* (MHOM/IR/03/Mash-878); Li: *L. infantum* (positive control); 55: infected *P. tobbi* to *L. infantum*; 5: *L. major* from positive human case; 8: *L. tropica* from positive human case; 11–13: *L. infantum* from positive human cases.

Of 80 females of *P. tobbi*, only 1 (1.25%) were found naturally infected with *L. infantum*. This is the first *L. infantum* DNA detection in *P. tobbi* in new focus Alborz province, northern Iran.

The ITS1 DNA sequences of this specimen were submitted to Genbank database with accession number (KC477100). Sequence comparison with other available data confirmed it as *L. infantum*. The specimen was identical (100%) or very similar to several *L. infantum* sequences deposited in Genbank, including isolates from Uzbekistan (Accession No. FN398341), Brazil (Accession No. FN398343) and France (Accession No. Aj634339). Also they were found to be 100% similar to *Leishmania chagasi* from Brazil (Accession No. AJ000304) and 99% similar to *L. donovani* from Bangladesh (Accession No. AB725909).

**Table 2**

Species composition frequency of sand flies in Savojbolagh County, Alborz Province, Northern Iran, 2012.

Species Villages	<i>P. sergenti</i>	<i>P. tobbi</i>	<i>P. caucasicus</i>	<i>P. papatasi</i>	<i>P. perfiliewi</i>	<i>P. kandelakii</i>	<i>P. major</i>	<i>P. alexandri</i>	Total
Arab Abad	229 (48.2%)	200 (42.1%)	15 (3.2%)	5 (1.1%)	9 (1.9%)	4 (0.8%)	11 (2.3%)	2 (0.4%)	475 (100%)
Hiv	132 (56.6%)	54 (23.2%)	25 (10.7%)	8 (3.4%)	12 (5.2%)	0 (0.0%)	2 (0.9%)	0 (0.0%)	233 (100%)
Aghasht	122 (61.3%)	46 (23.2%)	16 (8.0%)	3 (1.5%)	4 (2.0%)	7 (3.5%)	0 (0.0%)	1 (0.5%)	199 (100%)
Kordan	45 (35.4%)	41 (32.3%)	16 (12.6%)	6 (4.8%)	8 (6.3%)	2 (1.5%)	5 (3.9%)	4 (3.2%)	127 (100%)
Total	528 (51.1%)	341 (32.9%)	72 (7.0%)	22 (2.1%)	33 (3.2%)	13 (1.3%)	18 (1.7%)	7 (0.7%)	1034 (100%)

#### 4. Discussion

The ecology and epidemiology of Leishmaniasis are important measures for management and planning of disease control. Entomological surveys accompanied by epidemiological data provide essential information for the design of control programs of the disease.

Finding naturally infected wild-caught specimens that are anthropophilic fulfills two essential requirements for incriminating a sand fly vector<sup>[22]</sup>. In endemic areas where more than one *Leishmania* species is present, diagnostic tools are required for the detection of parasites directly in samples and distinguish all relevant *Leishmania* species<sup>[23]</sup>. Characterization of *Leishmania* species is important, because different species may require special remedial method. On the other hand, such information is also valuable in epidemiologic studies where the distribution of *Leishmania* species in hosts and insect vectors is an urgent item in the controlling programs<sup>[20,23]</sup>.

In many areas, however, despite considerable research on these diseases, the main ‘reservoir’ hosts and the species of sand fly responsible for transmission have yet to be identified. In many foci of VL, there is at least one species of sand fly that is common and anthropophilic to be considered as a probable vector, although good evidence to support this belief is lacking, such as the detection in wild caught females of this species and the parasites causing the VL. The prevalence of infection even in the primary vector may be quite low, making the detection of sand fly infection difficult and particularly the detection of any temporal trend in the prevalence of sand fly infection, especially, if dissection and microscopy constitute the detection method. Isoenzyme detection methods provide the gold-standard for identifying species and reference strains of *Leishmania*, but this method has disadvantage as it requires the culture of a large number of parasites and primary isolates can easily become contaminated, or a mixed infection can yield only the strain that grows fast in laboratory conditions<sup>[24]</sup>.

The highly sensitive technique of PCR has been used before to detect *Leishmania* in New and Old World sand flies<sup>[25,26]</sup>. Some species of sand flies belong to subgenus *Larroussius* are potential vectors of VL in the Mediterranean basin. Aransay *et al.* in 2000 used this method in Greece for detection of *Leishmania* infection in *Phlebotomus (Larroussius) neglectus*, *Phlebotomus (Larroussius) tobbi*, *Phlebotomus (Larroussius) simici*, and *Paraphlebotomus alexandri* using a semi-nested PCR technique<sup>[9,10]</sup>.

The main objectives of this study were to identify the sand fly vector(s) and the etiologic agents responsible for the recent cases of VL in rural areas of Savodjbolagh, which is a classic focus of *L. infantum*. In this study, only the species of *P. tobbi*, was infected with *L. infantum*. Previous studies in the country have revealed natural infection of *P. tobbi* with *L. infantum* in Ardebil province northeastern Iran<sup>[11]</sup>.

The vectorial competence of *P. tobbi* has been described previously in Cyprus, Greece, Turkey, Yugoslavia and Albania<sup>[27]</sup>. It has also been shown experimentally that, this species can support *L. infantum*. This species is distributed mainly in countries of the eastern and mid-northern

Mediterranean basin<sup>[27,28]</sup>. It is also one of the most important sand fly species in terms of public health in Greece and the proven vector of *L. infantum* in Cyprus<sup>[29,30]</sup>. As a final conclusion, *P. tobbi* was the only species found infected with *L. infantum*, so, it seems this species is playing the principal role in circulating of *L. infantum* between canine reservoirs and human in rural areas of Savodjbolagh district, Alborz province, north of Iran.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

The authors would like to appreciate very much the Health Center staff of Savodjbolagh district for their kind help. This study was financially supported by the School of Public Health, Tehran University of Medical Sciences (Project No. 17284).

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#### Comments

##### Background

VL is zoonotic and transmitted to humans by sand flies. Although three species of sand flies have been reported as the vector in north western and north eastern of Iran, no information has been reported on the vector of VL in the new focus of disease, northern Iran.

##### Research frontiers

Field studies were performed in order to determine sand fly vector of *Leishmania* parasites by using molecular biological techniques in Alborz, Iran. The authors found that *Phlebotomus tobbi* was the principal vector of *L. infantum* in rural areas of Savodjbolagh district, Alborz province, north of Iran.

##### Related reports

Previous studies in the country have revealed natural infection of *P. tobbi* with *L. infantum* in Ardebil province northeastern Iran. It is also one of the most important sand fly species in terms of public health in Greece and the proven vector of *L. infantum* in Cyprus.

##### Innovations & breakthroughs

This research was to identify the vector(s) of VL and species composition of sand flies were identified in the new focus of disease, northern Iran, using dissection and microscopic examination, as well as molecular techniques, including PCR of kDNA, ITS1-rDNA, followed by RFLP.

##### Applications

Combination techniques using nested-PCR amplification of kinetoplast DNA (kDNA), ITS1-rDNA, followed by *Hae* III digested RFLP are applicable for epidemiological field

research on identification of *Leishmania* parasites in sandflies.

#### Peer review

The authors could identify a sand fly vector of VL in the new focus of disease, northern Iran, using microscopic examination, as well as molecular techniques, including PCR of kDNA, ITS1-rDNA, followed by RFLP. The results may help the control of *P. tobbi* as a target vector in the area.

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