Natural infection of wild caught *Phlebotomus tobbi* to *Leishmania infantum* in East Azerbaijan province, northwestern Iran

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**ABSTRACT**

**Background & objectives:** Zoonotic visceral leishmaniasis is caused by *Leishmania infantum*, which is transmitted to humans by bites of phlebotomine sandflies and is one of the most important public health problems in Iran. To detect and identify the *Leishmania* parasites and their corresponding vector(s), an investigation was carried out in Azarshahr County, a new and important focus of the disease in East Azerbaijan province in northwestern Iran during late April to late October 2010.

**Methods:** Sandflies were sampled using sticky papers (A4 white paper soaked in castor oil) from inside and outside of the houses and animal shelters, close to the vegetation and crevices. The head and three last abdomen segments of the specimens were removed and mounted in Puri’s medium for species identification. The rest of body was subjected to molecular methods for detection of leishmanial parasites.

**Results:** Among 400 female sandflies tested by polymerase chain reaction (PCR) of kDNA, ITS1-rDNA, and CPB genes of the parasite followed by restriction fragment length polymorphism (RFLP), only 2 out of 8 *Phlebotomus tobbi* were positive to *L. infantum* parasites.

**Conclusion:** The results indicated that, *P. tobbi* was the only species found infected by *L. infantum* and the principal vector of the disease agent to human.

**Key words** Iran; *Leishmania infantum*; Phlebotomus; polymerase chain reaction; visceral leishmaniasis

**INTRODUCTION**

Leishmaniasis is a predominantly rural disease with multifaceted clinical manifestations caused by infections with species of *Leishmania*. The disease is prevalent in more than 88 countries in four continents. Leishmaniasis is considered as one of the neglected tropical diseases by the World Health Organization⁰. In general, the disease occurs in four major forms: cutaneous (CL), mucocutaneous, diffuse cutaneous, and visceral leishmaniasis (VL). More than 90% of VL cases in the world were reported from Bangladesh, Brazil, India, and Sudan². Zoonotic visceral leishmaniasis, which is commonly caused by *L. infantum* in the Mediterranean region, the Middle East, and Latin America, affects approximately half a million new patients each year³. In the Mediterranean basin, domestic dogs (*Canis familiaris*) are the principal reservoir hosts, and some species of sandflies belonging to the subgenus *Larroussius* are the primary vectors². Visceral leishmaniasis is the most severe form of leishmaniasis, which is nearly always fatal if left untreated⁴.

Sandfly species of the genus *Phlebotomus* are the only known vectors in the Old World⁵. Although Zoonotic VL occurs sporadically throughout Iran, there are four important endemic foci in the country: Ardebil and East Azerbaijan in the northwest, and Fars and Bushehr provinces in the south⁶. Infantile visceral leishmaniasis (IVL) caused by *L. infantum*, is highly endemic in some parts of northwest Iran: including Meshginshahr, Germi, Bilesar counties in Ardebil province as well as Azarshahr county in Eastern Azerbaijan province with high prevalence⁷–¹⁰. According to the records of Ministry of Health, 2056 kala-azar cases were reported between 1997 and 2006, of which 624 (30.4%) were from Ardebil province¹¹. Among the total of 54 (44 approved and 10 yet to be confirmed) sandflies species from Iran¹²–¹³, three species *Phlebotomus (Larroussius) kandelakii* Shchurenkova, *P. (Larroussius) perfiliewi transcaucasicus* Parrot and *P. tobbi* have been reported as vectors of *L. infantum* in the northwest of the country⁶,¹⁴–¹⁷. Also three species, *P. (Paraphlebotomus) alexandri* Sinton, *P. (Larroussius) major* Annandale, and *P. (Larroussius) keshishianii* Shchurenkova, have been suspected vectors of VL in south of Iran¹⁸–²¹. Wild and domestic carnivores are the main reservoirs, but rodents have been reported as reservoirs or accidental hosts in Iran²².
This study was carried out during late April to late October 2010 in rural areas of Azarshahr district, East Azerbaijan province, northwestern Iran, to identify the main vector(s) of *L. infantum*.

**MATERIAL & METHODS**

The investigation was carried out from late April to late October 2010 in 5 villages—Almalodash, Jaragil, Segaiesh, Amirdizaj and Germezigol in Azarshahr, Eastern Azerbaijan province, northwestern Iran. Azarshahr county (37°30′, 45°40′ E) is situated in the northwest of Iran, 54 km south of Tabriz at the altitude of 1368 m above the mean sea level. The mean annual precipitation is 303 mm with 49% relative humidity, and the mean annual temperature 15.2°C. The maximum and minimum average monthly temperatures were 27.7 and 3.7°C respectively. The total population of Azarshahr was 10,664 in 2009. The main occupation of the population is farming and raising animals.

**Sandfly collection and identification**

Sandflies were collected using sticky traps (Castor oil-coated white papers 20 cm × 30 cm) biweekly from indoors (bedrooms, guest room, toilet and table) and outdoors (rodent burrows). Traps (60 papers per village) were set at dusk and sandflies were collected at dawn. The sandfly 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 microtubes for DNA extraction. Specimens were mounted on glass slides using Puri’s medium and identified using the identification keys for species within Larrousius group23 and for species of other groups and subgenera24, 25.

**DNA extraction**

DNA was extracted by using the Bioneer Genomic DNA Extraction Kit. Extraction was carried out by grinding of individual sandfly 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), *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 (SPH-TUMS) were used as positive controls. DNA from *L. donovani* IPER/IR/2007/HS10 (isolated previously from sandflies was also used as a positive control18.

**Detection and identification of Leishmania species**

Initial screening of sandflies was performed by nested-PCR amplification of kinetoplast DNA (kDNA) using the primers (Table 1) and protocol described by Noyes *et al*26. 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 sec, 55°C for 60 sec, and 72°C for 90 sec. One microliter 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 the 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 HaeIII digestion of the resulting amplicons26, 27. 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 HaeIII without prior purification using conditions recommended by the supplier (Cinagen, Iran).

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_X28. Homologies with the available sequence data in GenBank were checked by using basic local alignment search tool (BLAST) analysis software (http://www.ncbi.nlm.nih.gov/BLAST). The sequences of *L. infantum* and *L. donovani* are almost identical in ITS1 region and discrimination of *L. infantum* from *L. donovani*.

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<td></td>
<td></td>
<td>CSB1XR</td>
<td>ATTTTCCGGCATTTGCCGAAAGC</td>
</tr>
<tr>
<td></td>
<td>Second</td>
<td>13Z</td>
<td>ACTGGGGGTTGGTGGTAGAAATAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LIR</td>
<td>TCAGCAGAACGCCCTT</td>
</tr>
<tr>
<td>ITS1</td>
<td>One</td>
<td>LITSR</td>
<td>CTTGACATTTTCCGATG</td>
</tr>
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<td></td>
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<td>TGATACCACTTATGCACCTT</td>
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<tr>
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<td>One</td>
<td>Forward</td>
<td>CGTGAACCGGGTGGAGAAT</td>
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25Oshaghi *et al*: Leishmaniasis vector(s) in Iran

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by the PCR-RFLP method\textsuperscript{29} is quite difficult. Hence, we
tried to use PCR-RFLP of CPBE/F gene followed by
DraIII digestion of the resulting amplicons\textsuperscript{18,30} to
discriminate the members of \textit{L. donovani} complex
(\textit{L. donovani} and \textit{L. infantum}) specimens that were PCR
positive against the ITS1 locus. The optimal conditions
for CPBE/F amplification in 30 µl were 6 pmol of each
primer (Table 1), 4.5 nmol dNTPs, 1 Unit Taq polymerase,
3 µl Buffer 10 and 1 µl of DNA extracted from individual
sandflies. A PCR protocol of 30 cycles of denaturation
30 s 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. Stan-
dard DNA fragments (100 bp ladder, Fermentas) were
used to permit sizing. PCR products (15 µl) of ITS1 and
CPBE/F were digested by \textit{Hae}III and DraIII respectively
without prior purification using conditions recommended
by the supplier (Cinagen, Iran). The restriction fragments
were subjected to electrophoresis in 2% agarose and vi-
ualized under UV light after staining for 15 min in
ethidium bromide (0.5 µg/ml). \textit{Hae}III digestion of ITS1
PCR revealed two fragments of 220 and 140 bp for \textit{L. major},
three fragments of 200, 80 and 60 bp for \textit{L. infantum/donovani},
and two fragments of 200 and 60 bp for \textit{L. tropica}. \textit{Dra}III enzyme cuts the CPBE/F amplicon
of \textit{L. donovani} into 400 and 341 bp fragments whereas
the CPBE/F PCR of \textit{L. infantum} remains intact.

RESULTS

In total, 5557 specimens comprising 16 species (14
\textit{Phlebotomus} and 2 \textit{Sergentomyia}) were collected and
identified. These included \textit{P. papatasi} (31.9%), \textit{P. sergenti}
(22.5%), \textit{P. mongolensis} (17.3%), \textit{P. ansarri} (0.3%), \textit{P.
tobbi} (3.4%), \textit{P. kandelakii} (4.2%), \textit{P. halepensis} (8.1%),
\textit{P. longiductus} (0.8%), \textit{P. anderjevi} (2.9%), \textit{P. caucasicus}
(5%), \textit{P. mesghali} (0.5%) \textit{P. perfiliewi transcaucasicus}
(0.5%), \textit{P. major} (0.5%), \textit{P. (adlerius)} sp (0.5%), \textit{S.
hodgsoni} (1.3%), \textit{S. pawlowskyi} (0.3%) (Table 2). The activities
of the species extended from April to October with a
single peak in August. Common specimens in resting
places were \textit{P. papatasi}, \textit{P. sergenti} and \textit{P. mongolensis}.

In all, 400 female sandflies including \textit{P. papatasi}
(50%), \textit{P. sergenti} (40%) \textit{P. major} (2%), \textit{P. perfiliewi
transcaucasicus} (3%), \textit{P. kandelakii} (3%) and \textit{P. tobbi}
(2%) were tested against the \textit{Leishmania} parasite genome.
Only 2 out of 8 specimens of \textit{P. tobbi} were positive for
\textit{L. infantum/donovani} using the nested PCR against kinetoplast DNA. This was observed in the kDNA nested-

Table 2. Species composition frequency of sandflies collected
in surveys conducted in 2010 in Azarshahr County,
East Azerbaijan, Iran

<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>Percent</th>
</tr>
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<tbody>
<tr>
<td>\textit{P. papatasi}</td>
<td>1773</td>
<td>31.9</td>
</tr>
<tr>
<td>\textit{P. sergenti}</td>
<td>1250</td>
<td>22.5</td>
</tr>
<tr>
<td>\textit{P. mongolensis}</td>
<td>961</td>
<td>17.3</td>
</tr>
<tr>
<td>\textit{P. ansarri}</td>
<td>17</td>
<td>0.3</td>
</tr>
<tr>
<td>\textit{P. tobbi}</td>
<td>189</td>
<td>3.4</td>
</tr>
<tr>
<td>\textit{P. kandelakii}</td>
<td>233</td>
<td>4.2</td>
</tr>
<tr>
<td>\textit{P. halepensis}</td>
<td>450</td>
<td>8.1</td>
</tr>
<tr>
<td>\textit{P. longiductus}</td>
<td>44</td>
<td>0.8</td>
</tr>
<tr>
<td>\textit{P. anderjevi}</td>
<td>161</td>
<td>2.9</td>
</tr>
<tr>
<td>\textit{P. caucasicus}</td>
<td>278</td>
<td>5</td>
</tr>
<tr>
<td>\textit{P. mesghali}</td>
<td>28</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{P. perfiliewi transcaucasicus}</td>
<td>28</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{P. major}</td>
<td>28</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{P. (adlerius)} sp</td>
<td>28</td>
<td>0.5</td>
</tr>
<tr>
<td>\textit{S. hodgsoni}</td>
<td>72</td>
<td>1.3</td>
</tr>
<tr>
<td>\textit{S. pawlowskyi}</td>
<td>17</td>
<td>0.3</td>
</tr>
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Total 5557 100

PCR amplification assays where a 680 bp PCR band was
produced. This length of PCR in the system is assigned
to \textit{L. infantum/L. donovani}. Examination of the two in-
fected specimens showed that their abdomens were empty.
Further analysis showed that these were positive against
ITS1 locus and produced a band of approximately 340
bp. Further, ITS1 PCR–RFLP analysis by \textit{Hae}III revealed
the fragments of 200, 80 and 60 bp for \textit{L. infantum} which
are characteristics of \textit{L. infantum/donovani}. The diagnostic
fragments are 220 and 140 bp for \textit{L. major} and 2 frag-
ments of 200 and 60 bp are for \textit{L. tropica} (Fig. 1).

Fig. 1: ITS1-rDNA PCR-RFLP analysis of selected strains of
\textit{Leishmania} species using \textit{Hae}III restriction enzyme. Lanes:
M–100 bp ladder (Fermentas); lane 1–\textit{L. major} (MHOM/IR/
75/ER: serial code of the standard \textit{L. major} species at the
reference laboratory of the Parasitology Department, Tehran
University of Medical Sciences; lane 2–\textit{L. tropica} (MHOM/
IR/03/Mash-878); lane 3–Infected \textit{Phlebotomus tobbi} to \textit{L.
infantum}; and lane 4–\textit{L. infantum} (positive control).
To discriminate between *L. infantum* and *L. donovani*, cpb PCR was performed and a 702 bp was produced for both specimens. Species-specific PCR of CPBE/F gene followed by DraIII RFLP analysis against the positive ITS1 rDNA specimens confirmed the presence of only *L. infantum* in two *P. tobbi* sandfly specimens. The enzyme could cut the 741 bp amplicon of *L. donovani* (positive control) into 400 and 341 bp fragments whereas the 702 bp of *L. infantum* remained intact. Of 8 female *P. tobbi*, 2 (25%) were found naturally infected with *L. infantum*. This is the first report of *P. tobbi* naturally infected with *L. infantum* in Azarshahr district a new focus of disease, East Azerbaijan province, northwestern Iran (Fig. 2).

**DISCUSSION**

In endemic areas, the effective control of the human leishmaniasis requires a thorough knowledge of the ecology and epidemiology of the parasites causing the diseases, and their vertebrate and invertebrate hosts. In many areas, however, despite considerable research on these diseases, the main ‘reservoir’ hosts and the species of sandfly responsible for transmission have yet to be identified. In many foci of VL, there is at least one species of sandfly 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 sandfly infection difficult and particularly the detection of any temporal trend in the prevalence of sandfly infection, especially, if dissection and microscopy constitute the detection method. *Leishmania*-specific PCR can provide an alternative method for the detection of *Leishmania* in naturally infected sandflies, that is highly sensitive yet less laborious than dissection and microscopy, does not require experienced microscopists, and can easily be used to screen pools of sandflies, rather than individual flies.

Control of leishmaniasis need to understand the ecology and epidemiology of disease in endemic areas. There is a major problem for epidemiologists both in identification of reservoir hosts and in detection of vectors. Therefore, finding naturally infected sandflies is essential in identifying a species as a vector of *Leishmania* and in studying infection rates in areas of endemcity.

The main objectives of this study were to identify the sandfly vector(s) and the etiologic agents responsible for the recent cases of VL in rural areas of Azarshahr, which is a classic focus of *L. infantum*. In this study, only the species of *P. tobbi*, were infected with *L. infantum*. Previous studies in the country have revealed natural infection of *P. tobbi* with *L. infantum* in Ardebil province adjacent to East Azerbaijan province, northeastern Iran. The species of *P. tobbi* belongs to the subgenus *Larroussius* and there are several vectors of *L. infantum* in this subgenus such as *P. (Larroussius) perfiliewi*, *P. (Lar.) neglectus*, *P. (Lar.) syriacus*, *P. (Lar.) major*, and *P. (Lar.) kandelakii* have been reported from the Eastern Mediterranean basin.

In this study, the prevalence of *P. tobbi* was 3.4% (Table 2) which is more than the prevalence rates (0.75, 2.8, 1.9, and 1.4%) previously reported from Turkey. The vectorial competence of *P. tobbi* has been described previously in Cyprus, Greece, Turkey, Yugoslavia and Albania. 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. It is also one of the most important sandfly species in terms of public health in Greece and the proven vector of *L. infantum* in Cyprus. 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 Azarshahr district, East Azerbaijan, northwestern Iran.

**ACKNOWLEDGEMENTS**

We are grateful to Mr Mohammad Reza ABAIE and Dr Ahmad Ali Hanafi bojd for their assistance in identifi-
cation of sandflies. This study received financial support from the School of Public Health, Tehran University of Medical Sciences, Project No. 10515, 2010.

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