Vector Incrimination of Sand Flies in the Most Important Visceral Leishmaniasis Focus in Iran

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Abstract. The prevalence, host preference, and rate of Leishmania spp. infection of sand fly species are important parameters for incrimination of parasite vectors. We applied polymerase chain reaction (PCR)–based and enzyme-linked immunosorbent assay (ELISA) methods to detect Leishmania spp. parasites and blood meals within individual sand flies in the most important visceral leishmaniasis (VL) focus in northwestern Iran. Leishmania spp. minicircles (kinetoplast DNA) were found in 14 (0.9%) of 1,569 female specimens. Sequence analysis of 650 basepairs of an internal transcribed spacer ribosomal DNA gene identified L. infantum/L. donovani in 12 specimens and L. adleri-like parasites in 2 specimens. Nine (64.3%) of 14 of the Leishmania spp.–positive sand flies were Phlebotomus perfeliewi transcaucasicus. Blood meal identification of host DNA within sand flies by PCR-based and ELISA methods showed that 30% and 28%, respectively, were positive for human blood. Results of this study showed that P. perfeliewi transcaucasicus is the most prevalent, infected, and anthropophagic sand fly and plays a major role in VL transmission in the region studied.

INTRODUCTION

Leishmaniasis is a serious health concern and its repercussions on public health and its geographic distribution, far from decreasing, appear to be increasing. The World Health Organization estimates that 1.5–2 million new cases occur each year.12 Twenty-two Leishmania spp. have been reported to cause human infections.3 In the Old World, cutaneous leishmaniasis (CL) is predominantly caused by L. major, L. tropica, L. aethiopica, and L. infantum, whereas L. donovani and L. infantum are responsible for visceral disease. Leishmaniasis causes a wide range of clinical forms ranging from a mere ulcer to a systemic disease that can be fatal if not treated correctly. Visceral leishmaniasis (VL) in Mediterranean region and Iran is caused by L. infantum, and it is considered a severe, often fatal disease.4 Domestic dogs (Canis familiaris) are the principal reservoir host, and some sand fly species of the genus Phlebotomus subgenus Larroussius are the primary vectors of VL in the region.5

There are three important endemic foci of VL in Iran: Ardebil, East Azerbaijan, and Fars provinces, and some sporadic foci.6 During 1998–2006, approximately 2,056 cases of VL were reported in Iran, and Ardabil Province contained approximately 30.4% of the cases. More than 90% of VL cases are reported in infants less than 10 years of age and domestic dogs are primary reservoir host of the disease.7 However, on the basis of entomologic studies, two species (P. kandelakii and P. perfeliewi transcaucasicus) are naturally infected with Leishmania spp. promasigotes in northwestern Iran and suspected as being probable vectors of VL in the region.8 Identification of reservoirs and vectors and determination of natural infection rates with Leishmania spp. parasites are important for definition of risk factors and epidemiologic control of leishmaniasis. However, the classic histopathologic and dissection methods used in these processes are time-consuming and are not always sufficiently sensitive for parasite species identification. Detection of parasites in vectors is difficult to carry out because the prevalence of parasites in vectors is usually low and its estimation requires a large number of sand flies to be dissected. For this reason, immunologic techniques based on serodeme or monoclonal antibodies have been developed for this purpose, but their usefulness for the characterization of species and strains is limited because of cross-reactivity with other phylogenetically similar organisms.9 Isoenzyme analysis and identification of distinct zymodemes has been used as a gold standard in studies of the epidemiology of leishmaniasis.10,11 Unfortunately, this technique requires isolation of the parasite, which is rarely conducted in routine diagnosis.

With advances in molecular techniques, a number of molecular markers and polymerase chain reaction (PCR) protocols have been developed to detect or identify small numbers of Leishmania spp. parasites.12 Amplification by PCR of fragments of repetitive DNA with diagnostic sizes or sequences have been used to identify Leishmania spp. in sand flies.13,14 Minicircles of kinetoplast DNA (kDNA) and nuclear ribosomal genes (rDNA) of Leishmania spp. are two main target genome sites for detection and identification of parasites.14,15 The kDNA contains approximately 10,000 minicircles of DNA 600–800 basepairs in size in this genus. The sequence of minicircles has been found to be characteristic of each species and strain of Leishmania spp.16 Most Leishmania spp. contain hundreds of tandemly repeated rDNA genes, which are believed to be homogeneous. This finding enables identification of train-specific markers.17 On the basis of variations in kDNA and rDNA genes in different species, species-specific primers have been developed for some Leishmania spp.10,19

The purpose of this study was to identify the main or probable vector(s) of VL in one of the main foci of the disease in northwestern Iran by 1) detection and identification of Leishmania spp. in sand flies using a semi-nested PCR for kDNA, a nested PCR for rDNA, and PCR-based sequencing of part of the internal transcribed spacer (ITS) region; and 2) determination of host preference (to identify the anthropophily rate) of sand flies by using PCR–restriction fragment length polymorphism (RFLP) analysis of the mitochondrial
(mt) DNA cytochrome B (cytB) gene and an enzyme-linked immunosorbert assay (ELISA).

MATERIALS AND METHODS

Study area. The study was conducted in Germi district, Meshgin-Shahr, Ardabil Province, in northwestern Iran. This region is 1,490 meters above the sea level. The total population of Germi was approximately 123,000 in 2002. The weather is hot (up to 40°C) in summer and cold (less than −20°C) in winter. The warm season is short (mid-May to mid-September). Annual rainfall is approximately 114 mm. The main occupations of the population are farming and raising animals.

On the basis of available epidemiologic data obtained from the Ministry of Health, local health authorities, and medical centers in Germi district, villages with higher incidence of VL were selected for the study. Three primary villages (Kalansora, Shah-Tapeh-si, and Hamzeh-Khanlo) were selected and were analyzed in an entomologic survey. Three secondary villages (Hasi-Kandy, Sarv-Aghaji, and Ghasem-Kandy) were also studied periodically.

Sample collection. Sticky traps were used to collect sand flies from human and animal dwellings, rodent and fox burrows, under bridges, and on the shores of rivers. Traps were set at dusk and flies were collected at dawn. A total of 150–200 sticky traps were set each day in each village. Sample collection began in early July and continued until late September when sand fly activity was reduced sharply. Sampling was carried out every three days in the primary villages and 1–3 times in the secondary villages.

Trapped sand flies removed from sticky papers with needles, washed with absolute ethanol, and transferred into microtubes filled with 96% ethanol. Tubes were kept frozen (−20°C) until species identification and DNA extraction. Of several thousand samples collected in the region, 300 blood-fed females were randomly selected for detection of blood meal within samples.

Identification of sand fly species. In the laboratory, samples were washed with detergent and double-distilled water, and heads and terminal abdomens of females were removed and mounted with Pouri solution on glass slides for diagnosis. For males, only heads were removed and mounted with Pouri solution on glass slides. Species were identified by using specific morphologic keys. Middle parts of female sand flies were placed in microtubes and kept frozen (−20°C) until DNA extraction.

Detection of Leishmania spp. in sand flies. DNA extraction and PCR amplification. After removal of spermatheca and heads of female sand flies, middle parts of bodies of each sample were individually subjected to DNA extraction by using the method of Ready and others.20 DNA from L. infantum provided to the Iran Institute of Pasteur by the World Health Organization was extracted and used as a positive control. The PCR amplification was carried out using genus-specific primers of kDNA in a semi-nested PCR according to Cupolillo and others.21 The PCR product was 1,124 base-pairs and contained part of 18S rDNA gene, part of the 28S rDNA gene, and all of ITS1, ITS2, and the 5.8S rDNA gene.

PCR sequencing. The ITS PCR products of sand flies infected with Leishmania spp. were subjected to agarose gel electrophoresis, purified from gels by using a gel purification kit, and sequenced (Seqlab, Göttingen, Germany). Sequences were checked to correct ambiguities. Homologies with the available sequence data in GenBank was checked by using basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST). Sequences were compared by alignment by using Clustal W software.22

Analysis of blood meals in sand flies. Three hundred blood-fed female sand flies were randomly selected from approximately 20,000 captured sand flies for blood meal identification. Sand flies were selected on the basis of location and capture sites to obtain a representative sample of sand flies in a region. We used two methods (serologic analysis and PCR) for blood meal identification. Samples were divided randomly into two groups; each group was analyzed by PCR-RFLP or ELISA.

Extraction of DNA from blood meals in sand flies. DNA extraction from blood-fed female, male, and unfed female sand flies and cow (used as a negative control) was conducted according to the procedure of Steiner and others.23 Samples were individually disrupted by mechanical homogenization in buffer containing 10 mM Tris-HCl, pH 8.0, 312.5 mM EDTA, 1% (w/v) sodium lauryl sarcosine, and 1% polyvinylpyrrolidone. Homogenates were heated to 90°C for 20 minutes and chilled on ice for 5 minutes. Samples were centrifuged at 13,000 × g for 5 minutes at room temperature. The supernatant was removed and diluted 20-fold in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

PCR amplification of the mtDNA cytB gene. Two regions of the mtDNA cytB gene were amplified for host blood meal identification of the blood-fed female specimens. For identification of human blood meals, a portion (358 basepairs) of the cytB gene was amplified and digested with Xho I.24 Sequences of primers used were 5'-CCATCCAACAT CTCAGCATGATGAAA-3' (forward) and 5'-CCCCTCAG AATGATATTGTCCCTCA-3' (reverse).25,26 The PCR amplifications were performed in 25 µL of a solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM deoxynucleotide triphosphates, 10 pmol of each primer, 1 unit of Taq DNA polymerase (Cinagene, Tehran, Iran), and 2.5 µL of DNA template solution. Samples were incubated at 95°C for 3.5 minutes; followed by 36 cycles at 95°C for 30 seconds, 58°C for 50 seconds, and 72°C for 40 seconds; and 72°C for 5 minutes.

To discriminate animal host blood meals, a second region of the mtDNA cytB gene was amplified by using the protocol of Kent and Morris.27 Sequences of forward and reverse primers were 5'-TGAGGACAATAATATCATTGAGG-3' (UNFOR403) and 5'-GGTTGTGCCTCAATCCATGTTA-3' (UNREV1025), respectively. Primers amplified a 623-basepair region of the cytB gene of vertebrate mtDNA. The PCR amplifications were performed in 25 µL of a solution containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin,
1.0 mM deoxynucleotide triphosphates, 0.5 units of Taq polymerase, 50 pmol of each primer, and 2.5 µL of extracted DNA. Samples were incubated at 95°C for 5 minutes; followed by 35 cycles at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute; and 72°C for 7 minutes. Products were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide. Electrophoresis was conducted using a GeneRuler 100-basepair molecular mass marker (Cinagene).

Sequence analysis for selection of restriction enzymes. Available sequences of the 623-basepair for human and probable vertebrate hosts (cow, goat, horse, ass, dog, and other Canidae) in the study area were obtained from GenBank and checked for species-specific restriction enzyme sites for each host DNA by using the Nebcutter program. Analysis showed that Hae III did not have a restriction site on human PCR products but it has various specific sites in PCR products of other vertebrates. This enzyme was selected for discrimination of the blood meal sources within sand flies.

Digestion of PCR products was performed in 25 µL of a solution containing 15 µL of PCR product mixed with 2.5 µL of enzyme buffers and 5 units of the restriction enzyme overlaid with two drops of mineral oil. The mixture was incubated at the temperature recommended by enzyme suppliers. An aliquot (14 µL) of the digestion product was mixed with 6 mL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol), loaded onto a 2.5% agarose gel, and subjected to electrophoresis. Gels were stained with ethidium bromide (2 mg/mL) and the RFLP profiles were visualized under ultraviolet light.

Serologic analysis. Analysis was performed by ELISA as described by Edrissian and others. The abdomen of blood-fed sand flies was dissected, placed in the well of a micro-ELISA plate (Nunc, Roskilde, Denmark), squashed with a glass rod, eluted with 50 µL of distilled water for 2 hours at room temperature. Fifty microliters of coating buffer (carbonate bicarbonate, pH 9.6) was then added to each well. Plates were washed three times with phosphate-buffered saline, Tween 20, pH 7.2. Fifty microliters of diluted goat anti-human IgG conjugated to alkaline phosphatase were added onto each well, incubated for 2 hours at 37°C, washed three times with phosphate-buffered saline, Tween 20, pH 7.2. One hundred microliters of substrate solution (1 mg/mL of p-nitrophenyl phosphate [Sigma, St. Louis, MO] in 10% diethanolamine buffer, pH 9.8, containing 0.5 mmol MgCl₂ and 0.02% NaN₃) was added to each well, and incubated in a dark chamber for 30 minutes at room temperature. Two wells that did not contain blood were used as negative controls and two wells that contained human blood were used as positive controls. Results were visually assessed, and absorbance was measured with an ELISA reader at 405 nm approximately 30 minutes after addition of substrate solution. The test well result was considered positive if a yellow color was observed.

RESULTS

Entomologic findings. We collected approximately 20,000 sand flies in this study. Approximately 3,000 male and female samples were selected for species identification and 1,569 female samples from this group were tested for infection with Leishmania spp. Morphologic analysis identified 13 species (10 Phlebotomus spp. and 3 Sergentomyia spp.) in the study area. Frequency, sex ratio, and species are shown in Table 1. Within the genus Phlebotomus, P. perffeliewi transcaucasicus was the most common (61%) and P. mongolensis was the least common (0.32%) species. Sex ratio (male:female) varied according to species; it was 1:2 for P. perffeliewi transcaucasicus and 1:97 for all species. Almost half (46.5%) of the samples were trapped in animal shelters, 13.5% inside houses, 27.2% in human-made places, and 12.8% in natural habitats. A total of 87.2% of sand flies were trapped in indoors or close to human houses or animals shelters. This finding suggests that sand flies are domestic and have adapted to be close to human or animal hosts. Abdominal examination of 1,569 females showed that 1,312 (83.6%) were empty, and 8.5%, 2.5%, and 5% were full of blood, semi-gravid, and gravid, respectively.

Detection of Leishmania spp. within sand flies. Kintoplast DNA semi-nested PCR. Semi-nested PCR amplification of the parasite minicircle kDNA from female sand flies showed that 14 (0.9%) of 1,569 specimens were infected with Leishmania spp. The PCR product was 720 basepairs, which is the expected length for L. infantum. Sand fly species infected were P. perffeliewi transcaucasicus, P. kandelaki, P. papatasi, (Adlerius) spp., and S. dentata. Nine (64.3%) of 14 infected sand flies were P. perffeliewi transcaucasicus.

Amplification of internal transcribed spacer rDNA. To identify Leishmania spp., the ITS region of the 14 infected

<table>
<thead>
<tr>
<th>Species</th>
<th>No. (%)</th>
<th>No. female (%)</th>
<th>No. infected (%)</th>
<th>Overall infection (%)</th>
<th>Leishmania species</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. perffeliewi transcaucasicus</td>
<td>2,099 (60.9)</td>
<td>954 (45)</td>
<td>9 (0.94)</td>
<td>0.57</td>
<td>L. infantum/L. donovani</td>
</tr>
<tr>
<td>P. papatasi</td>
<td>211 (6.1)</td>
<td>47 (22)</td>
<td>1 (2.13)</td>
<td>0.06</td>
<td>Not identified</td>
</tr>
<tr>
<td>P. sergenti</td>
<td>183 (5.3)</td>
<td>32 (17)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. tobbi</td>
<td>52 (1.5)</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. kandelaki</td>
<td>108 (3.1)</td>
<td>45 (42)</td>
<td>1 (2.22)</td>
<td>0.06</td>
<td>L. infantum/L. donovant</td>
</tr>
<tr>
<td>P. alexandri</td>
<td>53 (1.5)</td>
<td>1 (2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. mongolensis</td>
<td>11 (0.3)</td>
<td>1 (9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. (Adlerius) spp.</td>
<td>203 (5.9)</td>
<td>131 (69)</td>
<td>1 (0.76)</td>
<td>0.06</td>
<td>L. infantum/L. donovant</td>
</tr>
<tr>
<td>P. brevis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. halepensis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. longiductus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S. sintoni</td>
<td>186 (5.4)</td>
<td>44 (24)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S. dentata</td>
<td>340 (9.9)</td>
<td>313 (92)</td>
<td>2 (0.64)</td>
<td>0.13</td>
<td>L. adleri like</td>
</tr>
<tr>
<td>S. pavlovsky</td>
<td>1 (0.03)</td>
<td>1 (100)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>All species</td>
<td>3,447 (100)</td>
<td>1,569 (45)</td>
<td>14 (0.9)</td>
<td>0.9</td>
<td>–</td>
</tr>
</tbody>
</table>

*P = Phlebotomus; S. = Sergentomyia. Males of three species of the P. (Adlerius) subgenus (P. brevis, P. halepensis, and P. longiductus) were identified. However, females of these species are morphologically indistinguishable.
samples was amplified using universal primers. Thirteen of 14 samples were amplified and produced a 1,124-basepair product. The only sand fly species in which *Leishmania* spp. was not identified was *P. papatasi*. Products were purified and sequenced. Eleven of thirteen PCR products were sequenced. The sequencing was performed on the 3' end of the ITS region, which includes the 18S region, ITS2, and the 5.8S region. Sequences showed a length range of 644–660 basepairs. Comparison of sequences with available data in GenBank showed that all but one of the sequences were similar to the ITS of *L. infantum/L. donovani*. The sequences were submitted to Genbank under accession numbers EU637914–EU637924. One of the sequences was different from others and showed 67% similarity with *L. adleri*. This parasite was found in an *S. dentata* sand fly. The other 10 sequences were similar to each other and were identical at more than 95% positions. The polymorphic sites in sequences were due mostly to ambiguous or unclear nucleotides or unidentified gaps.

**Blood meal identification.** **PCR-RFLP.** DNA isolated from the blood-fed sand flies, positive controls (blood from a human and a cow), and negative controls (water, unfed female flies, and a male fly) were used as a template in a PCR. Most host DNAs were amplified, and negative controls yielded no PCR product. This result implied that only host, but not sand fly, DNA patterns were detected in amplified specimens.

DNA sequence analysis showed that the two regions of the mtDNA *cytB* gene digested with either *Xho* I or *Hae* III could distinguish human DNA in blood of blood-fed sand flies from the DNA of blood of other vertebrates. *Xho* I digested only the 358-basepair PCR product of human DNA and produced two bands (215 basepairs and 143 basepairs). Human DNA in blood had no restriction site present in the DNA of blood of other vertebrates. This finding was confirmed by results of digestion with *Hae* III. This enzyme did not digest the 623-basepair fragment of the *cytB* gene in DNA from human blood. However *Hae* III digested the equivalent DNA fragment from other vertebrates and could distinguish DNAs from cow, ass, goat, horse, dog, and other Canidae from each other (Figure 1). For example, *Hae* III produce two fragments (345 basepairs and 304 basepairs) from cow DNA, two fragments (552 basepairs and 70 basepairs) from Canidae DNA, and two fragments (170 basepairs and 453 basepairs) from goat DNA.

Results of PCR-RFLP analysis showed that 30% of the sand flies had fed on a vertebrate host: 52.5% on cows, 17.3% on humans and cows, 15.3% on dogs and cows, 10% on humans, 2.7% on humans and dogs, and 2.5% on dogs. Except for one specimen that contained *P. (Adlerius)* spp., all blood-fed specimens were *P. perfiliewi transcaucasicus*. Details of PCR-RFLP analysis are shown in Table 2. Results show that sand flies in...
TABLE 3
Female sand flies tested by enzyme-linked immunosorbent assay for human blood

<table>
<thead>
<tr>
<th>Capture site</th>
<th>No. (%) specimens</th>
<th>Female (%)</th>
<th>Phlebotomus (subgenus) species</th>
<th>No. of blood fed (%)</th>
<th>Human blood (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>464 (13.5)</td>
<td>191 (41)</td>
<td>Pheliphilus perfiliewi transcaucasicus</td>
<td>26 (13.6)</td>
<td>6 (23.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ph. (Adleri) spp.</td>
<td>7 (3.7)</td>
<td>2 (8.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pheliphilus transcaucasicus</td>
<td>31 (4.4)</td>
<td>14 (45.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. papatasi</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Animal</td>
<td>1,604 (46.5)</td>
<td>707 (44)</td>
<td>P. perfiliewi transcaucasicus</td>
<td>51 (11.5)</td>
<td>15 (29.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. (Adleri) spp.</td>
<td>20 (4.5)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. papatasi</td>
<td>1 (0.2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. kandelaki</td>
<td>3 (0.7)</td>
<td>0</td>
</tr>
<tr>
<td>Artificial</td>
<td>939 (27.2)</td>
<td>445 (47)</td>
<td>P. perfiliewi transcaucasicus</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Natural</td>
<td>440 (12.8)</td>
<td>226 (51)</td>
<td></td>
<td>141 (9.0)</td>
<td>37 (26.2)</td>
</tr>
<tr>
<td>Total</td>
<td>3,447 (100)</td>
<td>1,569 (45)</td>
<td></td>
<td>1,440 (42)</td>
<td>20 (28.6)</td>
</tr>
</tbody>
</table>

* Males of P. brevi, P. halephousis, and P. longiduccus of the P. (Adleri) subgenus were identified. However, females of these species are morphologically indistinguishable.

the study region are more zoophilic and prefer to feed on cows, humans, and dogs in that order.

ELISA. Serologic analysis by ELISA on 141 blood-fed sand flies showed that 37 (26.2%) specimens fed on humans (Table 3). Thirty-five of 37 human blood-fed samples were P. perfiliewi transcaucasicus. The two other seropositive samples in human blood were P. (Adleri) spp. Approximately 63% of these blood-fed sand flies were found in either human or animal shelters, which suggested that they were highly endophilic. Table 3 shows the frequency of the human seropositive samples in the villages studied. Results showed that more than half (57%) of the positive samples were found in Shah-Tapeh-si. All positive sand fly samples in this village were P. perfiliewi transcaucasicus.

**DISCUSSION**

This study showed that the primary vector of the VL in the Germi region is P. perfiliewi transcaucasicus. This species was the most prevalent and the most infected sand fly species in the region. It has a relatively high preference for feeding on humans. It frequently feeds on humans and rests in animal shelters until it lays eggs. The results are supported by those of a recent study in Kaleybar, one of the foci of VL in northwestern Iran, which are based on PCR detection and sequencing of parasite ITS regions of rDNA. Phlebotomus perfiliewi transcaucasicus was found to be the only vector of the causative agent of VL, found in that region.**30** Phlebotomus perfiliewi perfiliewi, the sister subspecies of P. perfiliewi transcaucasicus, has been identified as a vector of VL/CL caused by L. infantum in Mediterranean countries such as Italy and Algeria.**31,34**

In our study, we also found Leishmania spp. in P. kandelaki (0.06%) and P. (Adleri) spp. (0.06%). Also, some specimens of P. tobbi and P. alexandri were found in the study area. All of these species have been identified as vectors of VL in other areas,**5,38,39** and their role in transmission of disease should not be underestimated. However, because the prevalence of these species was not enough, they may play a weak role in the disease cycle in the region. Sargenomyia dentatae and P. papatasi were also found infected. However, because of specific interactions of Leishmania spp. parasites such as L. donovani/ L. infantum with sand fly species, these species are not able to support the life cycle of the parasite.**37**

Results of sequence comparisons of ITS regions of causative agents of VL in the study was similar to those for L. donovani or L. infantum. Analysis of sequence data available in Genbank showed that the ITS sequences of the L. donovani complex are similar and sometimes identical to each other. Although studies in Iran have shown that the causative of VL is L. infantum, the presence of L. donovani in neighboring countries (Turkey and Saudi Arabia) necessitates further studies to identify species that cause VL in the study region.**5,38**

Studies have shown that Leishmania spp. in sand flies could be successfully detected and identified by PCR methods.**14,14,30** In our study, although the incidence rate of VL was not high in 2006 (the number of reported cases in the region had decreased from more than 100 cases in 2000 to only 14 cases in 2006), we detected Leishmania spp. or Leishmania-like spp. in 14 sand flies from the region. Our study showed that PCR methods are relatively easy to perform and can process a large number of samples with limited effort compared with conventional microscopic examination after dissection. Thus, the PCR it will be a useful method for monitoring Leishmania spp. infection rates in sand fly populations and identifying prevalent Leishmania spp.

Our study has provided baseline information on VL in an active focus of this disease in Northwestern Iran. We identified sand fly species prevalent in the study area and primary and suspected vectors of VL, and addressed issues of anthropophily and endophily. These findings could be used in epidemiologic studies and strategic planning for the control of VL in the region.

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