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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 Organization[1]. 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 Sudan[2]. 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 year [3]. 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 vectors[2]. Visceral leishmaniasis is the most severe form of leishmaniasis, which is nearly always fatal if left untreated[4].

Sandfly species of the genus *Phlebotomus* are the only known vectors in the Old World[5]. 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 south[6]. Infantile visceral leishmaniasis (IVL) caused by *L. infantum*, is highly endemic in some parts of northwest Iran: including Meshginshahr, Germi, Bilesavar counties in Ardebil province as well as Azarshahr county in Eastern Azerbaijan province with high prevalence[7–10]. 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 province[11]. Among the total of 54 (44 approved and 10 yet to be confirmed) sandflies species from Iran[12–13], three species *Phlebotomus (Larroussius) kandelakii* Shchurenkova, *P. (Larrroussius) perfiliewi transcaucasicus* Parrot and *P. tobbi* have been reported as vectors of *L. infantum* in the northwest of the country[6,14–17]. 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 Iran[18–21]. Wild and domestic carnivores are the main reservoirs, but rodents have been reported as reservoirs or accidental hosts in Iran[22].

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 group[23] and for species of other groups and subgenera[24,25].

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 (SPHTUMS) were used as positive controls. DNA from *L. donovani*) IPER/IR/2007/HS10 (isolated previously from sandflies was also used as a positive control[18].

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 *Hae*III digestion of the resulting amplicons[26,27]. 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 *Hae*III 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_X[28]. 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*

Table 1. List and details of primers used in this study.

Target	PCR step	Name	Sequences (5'-3')
kDNA	First	CSB2XF	CGAGTA GCAGAAACTCCCGTTCA
		CSB1XR	ATTTTTCGCGATTTTCGCAGAACG
	Second	13Z	ACTGGGGGTTGGTGTAAAATAG
		LIR	TCGCAGAACGCCCCT
ITS1	One	LITSR	CTGGATCATTTTCCGATG
		L5.8S	TGATACCACTTATCGCACTT
CPB	One	Forward	CGTGACGCCGGTGAAGAAT
		Reverse	CGTGCACTCGGCCGTCTT

by the PCR-RFLP method[29] is quite difficult. Hence, we tried to use PCR-RFLP of CPBE/F gene followed by *Dra*III digestion of the resulting amplicons[18, 30] to discriminate the members of *L. donovani* complex (*L. donovani* and *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. Standard DNA fragments (100 bp ladder, Fermentas) were used to permit sizing. PCR products (15 µl) of ITS1 and CPBE/F were digested by *Hae*III and *Dra*III respectively without prior purification using conditions recommended by the supplier (Cinagen, Iran). The restriction fragments were subjected to electrophoresis in 2% agarose and visualized under UV light after staining for 15 min in ethidium bromide (0.5 µg/ml). *Hae*III digestion of ITS1 PCR revealed two fragments of 220 and 140 bp for *L. major*, three fragments of 200, 80 and 60 bp for *L. infantum/donovani*, and two fragments of 200 and 60 bp for *L. tropica*. *Dra*III enzyme cuts the CPBE/F amplicon of *L. donovani* into 400 and 341 bp fragments whereas the CPBE/F PCR of *L. infantum* remains intact.

RESULTS

In total, 5557 specimens comprising 16 species (14 *Phlebotomus* and 2 *Sergentomyia*) were collected and identified. These included *P. papatasi* (31.9%), *P. sergenti* (22.5%), *P. mongolensis* (17.3%), *P. ansarii* (0.3%), *P. tobbi* (3.4%), *P. kandelakii* (4.2%), *P. halepensis* (8.1%), *P. longiductus* (0.8%), *P. anderjevi* (2.9%), *P. caucasicus* (5%), *P. mesghali* (0.5%) *P. perfiliewi transcaucasicus* (0.5%), *P. major* (0.5%), *P.* (*adlerius*) sp (0.5%), *S. hodgsoni* (1.3%), *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 *P. papatasi, P. sergenti* and *P. mongolensis.*

In all, 400 female sandflies including *P. papatasi* (50%), *P. sergenti* (40%) *P. major* (2%), *P. perfiliewi transcaucasicus* (3%), *P. kandelakii* (3%) and *P. tobbi* (2%) were tested against the *Leishmania* parasite genome. Only 2 out of 8 specimens of *P. tobbi* 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 two infected 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 *Hae*III revealed the fragments of 200, 80 and 60 bp for *L. infantum* which are characteristics of *L. infantum/donovani*. The diagnostic fragments are 220 and 140 bp for *L. major* and 2 fragments of 200 and 60 bp are for *L. tropica* (Fig. 1).

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

Species	No.	Percent
P. papatasi	1773	31.9
P. sergenti	1250	22.5
P. mongolensis	961	17.3
P. ansarii	17	0.3
P. tobbi	189	3.4
P. kandelakii	233	4.2
P. halepensis	450	8.1
P. longiductus	44	0.8
P. anderjevi	161	2.9
P. caucasicus	278	5
P. mesghali	28	0.5
P. perfiliewi transcaucasicus	28	0.5
P. major	28	0.5
P. (adlerius) sp	28	0.5
S. hodgsoni	72	1.3
S. pawlowskyi	17	0.3
Total	5557	100

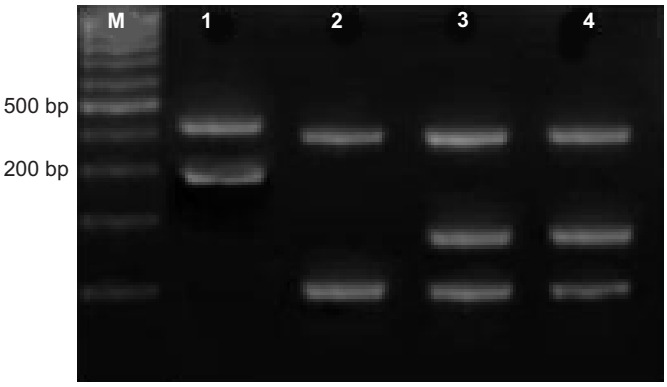

Fig. 1: ITS1-rDNA PCR-RFLP analysis of selected strains of *Leishmania* species using *Hae*III restriction enzyme. Lanes: M–100 bp ladder (Fermentas); lane 1–*L. major* (MHOM/IR/75/ER: serial code of the standard *L. major* species at the reference laboratory of the Parasitology Department, Tehran University of Medical Sciences; lane 2– *L. tropica* (MHOM/IR/03/Mash-878); lane 3–Infected *Phlebotomus tobbi* to *L. infantum*; and lane 4–*L. infantum* (positive control).

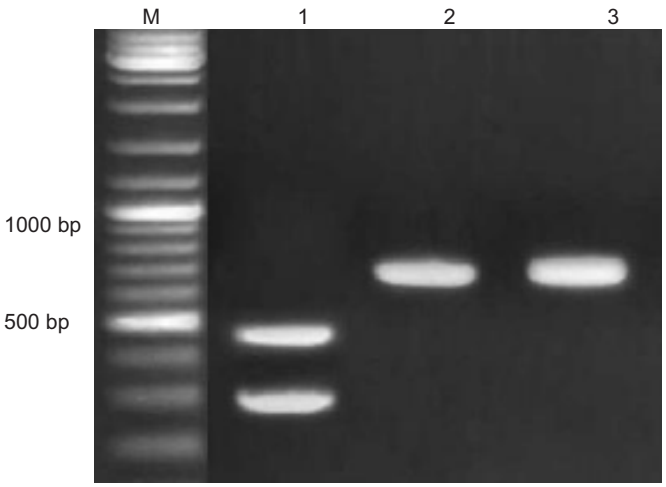

Fig. 2: cpbE/F gene PCR-RFLP analysis of selected strains of *Leishmania donovani* complex with the use of the restriction enzyme *Dra*III. Lanes: M–100 bp ladder (Fermentas); lane 1–*L. donovani*: cpbF (IPER/IR/2007/HS10); and lanes 2 & 3–*L. infantum* from infected *Phlebotomus tobbi.*

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 *Dra*III 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[31]. 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[32–35].

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 endemicity[36,37].

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[15]. 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[38]. The vectorial competence of *P. tobbi* has been described previously in Cyprus, Greece, Turkey, Yugoslavia and Albania[39]. It has also been shown experimentally that this species can support *L. infantum*[40]. This species is distributed mainly in countries of the eastern and mid-northern Mediterranean basin[39]. It is also one of the most important sandfly species in terms of public health in Greece[40] and the proven vector of *L. infantum* in Cyprus[41–42]. 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.

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REFERENCES

1. *First WHO report on neglected tropical diseases 2010*. Working to overcome the global impact of neglected tropical diseases. Geneva: World Health Organization 2010; p.1–169.
2. Lachaud LS, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J Clin Microbiol* 2002; *40:* 210–5.
3. *Control of the Leishmaniases*. Report of a WHO Expert Committee. *Tech Rep Ser 1990/ 793*. Geneva: World Health Organization 1990; p.1–158
4. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol* 2002; *9:* 951–8.
5. Alexander B, Maroli M. Control of phlebotomine sandflies. *Med Vet Entomol* 2003; *17:* 1–18.
6. Rassi Y, Javadian E, Nadim A, Rafizadeh S, Zahraii A, Azizi K, *et al*. *Phlebotomus perfiliewi transcaucasicus*, a vector of *Leishmania infantum* in northwestern Iran. *J Med Entomol* 2009; *46:* 1094–8.
7. Nadim A, Javadian E, Tahvildari-Bidruni Gh, Mottaghi M, Abai MR. Epidemiological aspects of kala-azar in Meshkin-Shahr, Iran: Investigation on vectors. *Iran J Public Health* 1992; *21:* 61–72.
8. Gavgani AS, Hodjati MH, Mohite H, Davies CR. Effect of insecticide impregnated dog collars on incidence rate of zoonotic visceral leishmaniasis in Iranian children: A matched-cluster randomised trial. *Lancet* 2002; *360:* 374–9.
9. Gavgani AS, Mohite M, Edrissian H , Mohebali M, Davies CR. Domestic dog ownership in Iran is a risk factor for human infection with *Leishmania infantum. Am J Trop Med Hyg* 2002; *67:* 511–5.
10. Mohebali M, Hajjaran H, Hamzavi Y, Mobedi I, Arshi SH, Zarei Z, *et al.* Epidemiological aspects of canine visceral leishmaniasis in the Islamic Republic of Iran. *Vet Parasitol* 2005; *129:* 243–51.
11. *Annual Report on Leishmaniasis in Iran.* Tehran: Ministry of Health and Medical Education 2007; p. 1–60.
12. Kasiri H, Javadian E, Seyedi-Rashti MA. Liste des Phlebotominae (Diptera: Psychodidae) d'Iran. *Bull Soc Pathol Exot* 2000; *93:* 129–30.
13. Rassi Y, Hanafi-Bojd AA. Phlebotomine sandflies, vectors of Leishmaniases: Morphology, biology, ecology, and field and laboratory methods with pictorial key of Iranian sandflies. Tehran, Iran: Noavaran-Elm Publication 2006; p.1– 251.
14. Rassi Y, Javadian E, Nadim A, Zahraii AR, Vatandoost H, Motazedian H, *et al*. *Phlebotomus (Larroussius) kandelakii* the principal and proven vector of visceral leishmaniasis in northwest of Iran. *Pakistan J Biol Sci* 2005; *8:* 1802–6.
15. Rassi Y, Sanei Dehkordi A, Oshaghi MA, Abaie MR, Mohtarami F, Enayati A, *et al*. First report on natural infection of the *Phlebotomus tobbi* by *Leishmania infantum* in northwestern Iran. *Exp Parasitol* 2012; *131:* 344–9.
16. Oshaghi MA, Maleki Ravasan N, Hide M, Javadian E, Rassi Y, Sadraei J, *et al*. *Phlebotomus perfiliewi transcaucasicus* is circulating both *Leishmania donovani* and *L. infantum* in northwest Iran. *Exp Parasitol* 2009; *123:* 218–25.
17. Oshaghi MA, Maleki Ravasan N, Hide M, Javadian E, Rassi Y, Sadraei J, *et al*. Vector incrimination of sandflies in the most important visceral leishmaniasis focus in Iran. *Am J Trop Med Hyg* 2009; *81:* 572–7.
18. Oshaghi MA, Ravasan NM, Hide M, Javadian EA, Rassi Y, Sedaghat MM, *et al.* Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for *L. infantum/L. donovani* discrimination. *Exp Parasitol* 2009; *122*(1): 61–5.
19. Sahabi Z, Seyyedi-Rashti MA , Nadim A, Javadian E, Kazemeini M, Abai MR. A preliminary report on the natural leptomonad infection of *Phlebotomus major* in an endemic focus of VL in Fars province, south of Iran. *Iran J Public Health* 1992; *21:* 87–93.
20. Seyyedi-Rashti MA, Sahabi Z , Kanani-Notash A. *Phlebotomus (Larroussius) keshishiani* Shchurenkova 1936, another vector of visceral leishmaniasis in Iran. *Iran J Public Health* 1995; *24:* 23–30.
21. Azizi K, Rassi Y, Javadian E, Motazedian MH, Rafizadeh S, Yaghoobi-Ershadi MR, *et al*. *Phlebotomus (Paraphlebotomus) alexandri*: A probable vector of *Leishmania infantum* in Iran. *Ann Trop Med Parasitol* 2006; *100:* 63–8.
22. Edrissian GH, Nadim A, Alborzi AV, Ardehali S. Visceral leishmaniasis: The Iranian experiences. *Arch Iran Med* 1999; *1:* 22–6.
23. Absavaran A, Rassi Y, Parvizi P, Oshaghi MA, Abaie MR, Rafizadeh S, *et al*. Identification of sandflies of the subgenus *Larroussius* based on molecular and morphological characters in northwestern Iran. *Iran J Arthropod-Borne Dis* 2009; *3:* 22–35.
24. Theodor O, Mesghali A. On the Phlebotominae of Iran. *J Med Entomol* 1964; *1:* 285–300.
25. Lewis DJ. A taxonomic review of the genus *Phlebotomus*. *Bull Br Mus Nat Hist Entomol* 1982; *45:* 121–209.
26. Noyes HA, Reyburn H, Bailey JW, Smith D. A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast mini circle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan. *J Clin Microbiol* 1998; *36:* 2877–81.
27. El Tai NO, Osman OF, El Fari M, Presber W, Schönian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans R Soc Trop Med Hyg* 2000; *94:* 575–9.
28. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* 1997; *25:* 4876–82.
29. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, *et al*. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis* 2003; *47:* 349–58.
30. Hide M, Bañuls AL. Species-specific PCR assay for *L. infantum/ L. donovani* discrimination. *Acta Trop* 2006; *100:* 241–5.
31. Aransay AM, Scoulica E, Tselentis Y. Detection and identification of *Leishmania* DNA within naturally infected sandflies by seminested PCR on minicircle kinetoplastic DNA. *Appl Environ Microbiol* 2000; *66:* 1933–8.
32. Ready PD, Smith DF, Killick-Kendrick R. DNA hybridization on squash-blotted sandflies to identify both *Phlebotomus papatasi* and infecting *Leishmania major*. *Med Vet Entomol* 1988; *2:* 109–16.
33. de Bruijn MH, Barker DC. Diagnosis of New World leishmania-

sis: Specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. *Acta Trop* 1992; *52:* 45–58.
34. Mukherjee S, Hassan MQ, Ghosh A, Ghosh KN, GThosh KNBhattacharya A, Adhya S. *Leishmania* DNA in *Phlebotomus* and *Sergentomyia* species during a kala-azar epidemic. *Am J Trop Med Hyg* 1997; *57:* 423–5.
35. Rodriguez N, Aguilar CM, Barrios MA, Barker DC. Detection of *Leishmania braziliensis* in naturally infected individual sand flies by the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 1999; *93:* 47–9.
36. Killick-Kendrick R. Phlebotomine vectors of the leishmaniases: A review. *J Med Vet Entomol* 1990; *4:* 1–24.
37. Sharma U, Singh S. Insect vectors of *Leishmania*: Distribution, physiology and their control. *J Vector Borne Dis* 2008; *45:* 255–72.
38. Svobodová M, Alten B, Zídková L, Hlavac_ková J, Myšková J, Šeblová V, *et al* Cutaneous leishmaniasis caused by *Leishmania infantum* transmitted by *Phlebotomus tobbi*. *Int J Parasitol* 2009; *39:* 251–6.
39. Ready PD. Leishmaniasis emergence in Europe. *Euro Surveill* 2010; *15*: 19505. Available from: *http://www.eurosurveillance.org/ViewArticle.aspx?Article ID=19505.*
40. Killick-Kendrick R. The biology and control of phlebotomine sandflies. *Clin Dermatol* 1999; *17:* 279–89.
41. Mazeris A, Soteriadou K, Dedet JP, Haralambous C, Tsatsaris A, Moschandreas J, *et al*. Leishmaniases and the Cyprus Paradox. *Am J Trop Med Hyg* 2010; *82*(3): 441–8.
42. Léger N, Depaquit J, Ferté H, Rioux JA, Gantier JC, Gramiccia M, *et al.* Phlebotomine sandflies (Diptera: Psychodidae) of the isle of Cyprus. II. Isolation and typing of *Leishmania (Leishmania infantum)* Nicolle, 1908 (zymodeme MON 1) from *Phlebotomus (Larroussius) tobbi* Adler and Theodor 1930. *Parasite* 2000; *7:* 143–6.

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