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Occurrence of low density of *Leishmania infantum* in sandflies from a new focus of visceral leishmaniasis in northwest of Iran

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ABSTRACT

Background & objectives: Observations and case studies have shown that the number of Visceral Leishmaniasis (VL) cases have increased in the recent years in several areas of Iran including Sarab district, East Azerbaijan province. Sarab district has been considered as a new focus of VL in Iran. The density of the sandfly vector and the *Leishmania* parasites causing infection has been assessed in 2009.

Methods: Sandfly species had been collected from Sarab district, East Azerbaijan province in 2009 using sticky papers and CDC traps. DNA of sandflies was extracted and nested PCR was amplified in a region of the ribosomal RNA amplicon of *Leishmania* (ITS1-5.8S *rRNA* gene), which was shown to be species-specific by DNA sequence.

Results: Altogether, 1317 male and female sandflies were trapped. At least 10 different sandfly species were identified morphologically. *Leishmania infantum* was the only *Leishmania* that was detected among the sandfly's population in Sarab district. All the infectious cases (4/223) found in the abundant sandfly region were *Phlebotomus kandelakii.*

Conclusion: The diversity of sandflies was similar to those in the main VL focal points in Iran, but the diversity of parasite and density were significantly lower. The low prevalence of VL in Sarab district might be explained by the scarcity of infected domestic dogs *Canis familiaris* the primary reservoir host of VL in the region. By finding the *L. infantum* in *P. kandelakii* for the first time on this new focus, we are able to conclude that *P. kandelakii* might be the vector of *L. infantum*. In future, more works should be done to test status of *P. kandelakii* as a proven vector of *L. infantum.*

Key words *Leishmania infantum*; northern Iran; *Phlebotomus kandelakii;* visceral leishmaniasis

INTRODUCTION

Visceral leishmaniasis (VL) is a parasitic disease caused by infection with protozoan parasites of the *Leishmania infantum* and *L. donovani* (Kinetoplastidae: Trypanosomatidae) that are transmitted by the bites of phlebotomine sandflies (Diptera: Psychodidae) of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World[1–3]. Some of the most important foci of VL in the world are located in the mediterranean littoral, central Asia, Africa, south and central America[4].

Visceral leishmaniasis in mediterranean region and Iran is caused by *L. infantun*, and it is considered a severe, often fatal disease. Domestic dogs (*Canis familiaris*) are the principle reservoir host, and some sandfly species of the genus *Phlebotomus* subgenus *Larroussius* are the primary vectors of VL in the region[3, 4].

There are three important endemic foci of VL in Iran: Ardabil province, east Azerbaijan province both in north of Iran, also in Fars province in south of Iran, and some sporadic foci[5]. More than 50 species belonging to the *Phlebotomus* and *Sergentomyia* genera have been reported from Iran with some of these species being proven or probable vectors of human leishmaniasis in the Old World[1, 6]. On the basis of entomologic studies, two species of *Phlebotomus kandelakii* and *P. perfilliewi* transcaucasicus were naturally infected with *Leishmania* promasigotes in Iran[7, 8]. Our study sites are closed to part of an important VL focus in east Azerbaijan province, in north of Iran (Fig. 1).

During 1998–2006, approximately 2056 cases of VL were reported in Iran of which 30.4% of the cases were reported in Ardabil province, where it bordered our study site (Fig.1). More than 90% of VL cases are reported in infants <10 yr of age and domestic dogs are primary reservoir host of the disease[9]. The objective of this study was to identify sandfly species, fauna, and distribution of *Leishmania* infections in sandflies and also to assess the role of which sandfly species is a vector in the transmission of the disease to humans.

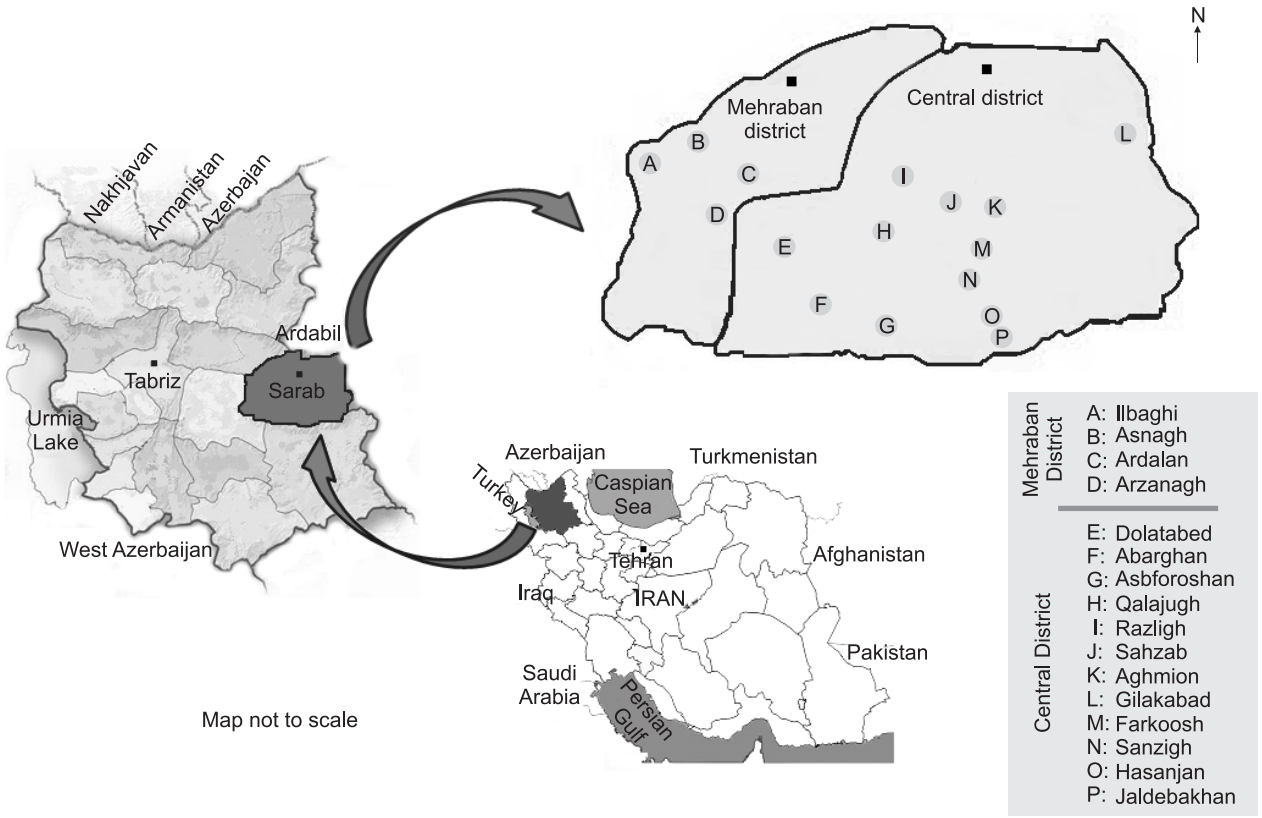

Fig. 1: Locations of the named foci of visceral leishmaniasis in northwest of Iran in Sarab, East Azerbaijan province of Iran.

MATERIAL & METHODS

The sandfly collections were carried out in 2009 during the activity of adult sandflies, in 16 villages at two locations in Sarab district, East Azerbaijan province (Fig. 1, Table 1). The villages sampled for current investigations were included from central district and Mehraban district (1610 m above sea level (m a.s.l.) (33° 51′ N and 31° 41′ E) within the known focus of VL reported by the regional public health services for interventions.

Sandflies were collected on sticky papers (A4 sheets of white paper soaked in castor oil) placed overnight in ruined outhouses, inside houses, domestic animal shelters, gaps on stony walls and at the entrances to gerbil burrows (1 paper per burrow; papers: 2–10 m apart, 30–40 per site, over a range of 120–200 m at each site). CDC miniature light traps (with the white-light bulb 1–2 m above ground level) were set overnight to sample sandflies in domestic animal shelters (1–2 traps per site), and a manual aspirator was sometimes used by a single collector to capture sandflies resting inside houses in the morning hours.

Sandflies captured in light traps were narcotized with cigarette smoke, and those caught on sticky papers were removed with needles or fine brushes dipped in 70% ethanol. All the sandflies were identified based on external and internal morphological characters of the head and abdominal terminalia which were slide-mounted in Berlese fluid following dissection with sterilized forceps and micro-needles[10]. The genomic DNA of each sandfly and any parasite within it was extracted and this was done within the molecular biology laboratory suite in a room where amplified and cloned DNAs were never processed[11].

The female sandflies from these collections were screened for infections of *Leishmania* species by nested PCR of a fragment of ITS-rDNA. Each PCR was carried out in two separate tubes. The first-stage PCR used the forward primer IR1 (at the 3′ end of the small subunit *rRNA* gene) with the reverse primer IR2 (at the 5' end of the large subunit *rRNA* gene). In the second-stage PCR: 1) the ITS1-5.8S fragment was amplified, using the nested forward primer ITS1F (overlapping the 3′ end of the small subunit *rRNA* gene and ITS1) with the nested reverse primer ITS2R4 (at the 5' end of ITS2)[11].

PCR products of fragment were directly sequenced on each strand to identify *Leishmania* haplotypes infecting individual sandflies. For this, individual contig sequences were aligned with GenBank sequences of all regional species using Sequencer 4.4 software for PC (Gene

Codes Corp, Ann Arbor, Michigan, USA), and unique haplotypes were identified after export into MEGA software for phylogenetic analysis[12].

RESULTS

Leishmania infections of sandflies identified by ITS-rDNA haplotypes

For the vector incrimination, female sandflies were screened for *Leishmania* infections using samples collected from different habitats and nearby domestic animal shelters in Sarab (north Iran). Sandflies from this location were came from foci of VL, in which the *Phlebotomus larrossious* subgenus species is believed to be the vectors[1, 2, 11]. By using nested PCR of ITS-rDNA (nested primer-pair ITS1F and ITS2R4), *L. infantum* were found only in *P. kandelakii* (4/223 infections). Two out of 4, *P. kandelakii* with *L. infantum* positive were gravid and two semi-gravid. One haplotype of *L. infantum* was identified from Sarab in female sandflies. A common haplotype (EU604810) of *L. infantum* was found in *P. kandelakii* from four different villages in Sarab (Table 1).

Sandflies collection, identification and fauna

The collections of sandflies contained the putative vectors of *L. infantum* causing VL in Iran (Tables 1 & 2).

Table 1. Prevalence of *Leishmania* positive using PCR assay of ITS-rDNA based on habitats, traps and date of collection among female *P. kandelakii* collected from Villages of Sarab, Iran

Collection site		Date of collection	Abdomen				Trap type		Habitat					Total PCR screened	PCR *Leishmania* (+)ve
Sarab	Villages		UF	FF	SG	G	CDC	SP	RB	ASH	Home	Store	Yard		
	Abarghan	18.05.2009	2	1	1	1	3	2		5				5	
	Ardalan	18.05.2009	4	1	1	3	6	3		8	1			9	
		16.06.2009				1		1		1				1	
		6.07.2009				4		4		3			1	4	
		9.08.2009		2		1(1)		3		3				3	1
	Arzanagh	14.06.2009			1			1		1				1	
		6.07.2009				2		2		1	1			2	
		10.08.2009			1	1		2					2	2	
	Aghmion	8.07.2009				1		1		1				1	
	Asbforoshan	18.05.2009	9			1	10			10				10	
	Asnagh	18.05.2009	4					4		2			2	4	
	Dolatabad	6.07.2009			1	6(1)		7		7				7	1
		10.08.2009		1		1		2		2				2	
	Farkoosh	18.05.2009	4	1	2		2	5		7				7	
	Gilakabad	18.05.2009	1			1		2		2				2	
		10.08.2009		1	1	1		3		3				3	
	Hasanjan	5.07.2009				7		7		1	6			7	
	Ilbaghi	18.05.2009	4		24	1	28	1	1	28				29	
	Jaldebakhan	5.07.2009				2		2		1			1	2	
		9.08.2009		1	4(1)	2		7		5	1		1	7	1
	Qalajugh	15.06.2009			3(1)			3		3				3	1
		6.07.2009			1			1		1				1	
		9.08.2009				2		2			1	1		2	
	Razligh	18.05.2009	6	1	1		1	7		6			2	8	
		15.06.2009			1			1		1				1	
		6.07.2009				7		7		7				7	
	Sahzab	18.05.2009	1	1				2		2				2	
		5.07.2009				2		2					2	2	
	Sanzigh	18.05.2009	79	2	2	2	2	83		84		1		85	
		14.06.2009		1				1		1				1	
		5.07.2009				2		2		1	1			2	
		10.08.2009			1			1			1			1	
	Total		114	13	45	51	52	171	1	197	12	2	11	223	4
			223				223		223						

ASH = Animal shelters, inside houses and yards; RB = Rodent borrow; SP = Sticky paper; CDC = Center for Disease Control, miniature light-traps; UF = Unfed; F= Fresh fed; SG = Semi-gravid; G = Gravid; Figures in parentheses indicate *Leishmania* positive.

Table 2. Distribution of sandflies in different villages of Sarab district, East Azerbaijan, Iran

Sub genus	*Larrossious*								*Adlerius*					*Paraphlebotomus*		*Phlebotomus*		*Sergentomyia*	Total
Species	*P. kandelakii*		*P. perfilliewi*		*P. major*		*P. tobbi*		*P. simici*	*P. brevis*	*P. balcanicus*	*P. halepensis*	*Adlerius* sp	*P. sergenti*		*P. papatasi*		s.s.p	
Sex	M	F	M	F	M	F	M	F	M	M	M	M	F	M	F	M	F		
Villages																			
Abarghan	5	5																	10
Ardalan	44	30	12	10	4	3	2	2	2			3	3	11	12	6	4	8	156
Arzanagh	11	5	1	9		1	5	7		1		5	5	8	8	7	3	8	84
Aghmion	4	1	1		1				1				2						10
Asbforoshan	18	10																	28
Asnagh	14	4																	18
Dolatabad	16	13	5	6	5	2	7	1				1	2	9	12	5	7	5	96
Farkoosh	2	7																	9
Gilakabad	8	12	2	5	1		7		1	1	1	7	8	3	5	5	1	8	75
Hasnjan	11	16	11		5	5	2	6			1	4	3	11	4	8	13	8	108
Ilbaghi	4	29				4							2						39
Jaldebakhan	5	15	12	2	9			2				7	5	12	7	5	2	4	87
Qalajugh	18	13	3	4	4	3	1	1	3	1	1	1	4	13	13	5	7	11	106
Razligh	28	21		9		1	4	6	2			3	2	14	3	3	4	11	111
Sahzab	15	10	7	7	4	7	6	5		1		5	3	6	17	5	4	10	112
Sanzigh	62	100	6	9	6	4	11	3	1		1	7	5	12	14	7	7	13	268
Trap type	CDC SP	(114) (442)	CDC SP	(19) (102)	CDC SP	(11) (58)	CDC SP	(14) (64)	CDC (0) SP (10)	CDC (1) SP (3)	CDC (0) SP (4)	CDC (9) SP (34)	CDC (16) SP (28)	CDC SP	(37) (157)	CDC SP	(20) (88)	CDC (13) SP (73)	
Total = 1317	265	291	60	61	39	30	45	33	10	4	4	43	44	99	95	56	52	86	1317
Percent	556 (42.2%)		121 (9.2%)		69 (5.2%)		78 (6.0%)		10 (0.7%)	4 (0.3%)	4 (0.3%)	43 (3.3%)	44 (3.3%)	194 (14.7%)		108 (8.2%)		86 (6.5%)	

M: Males; F: Females.

Altogether, 1317 adult sandflies from two districts (16 villages) in Sarab, East Azerbaijan province were collected in May to August 2009 (Table 2). The male and female sandflies were sampled during adult activity seasons.

In all, 10 species of genus *Phlebotomus* sandflies were trapped and identified morphologically. In addition, 86 sandflies belong to *Sergentomyia* genus were collected but the species were not identified. Four species belonged to *Larrossious* subgenus including *P. kandelakii* (total 556 sandflies: males 265 and females 291), *P. perfilliewi* (total 121: males 60 and females 61), *P. major* (total 69: males 39 and females 30), and *P. tobbi* (total 78: males 45 and females 33); and four species were belonged to *Adlerius* subgenus including *P. simici*, (total 10: males 10 and female 0), *P. brevis* (total 4: males 4 and female 0), *P. balcanicus* (total 4: males 4 and female 0) and *P. halepensis* (total 43: males 43 and female 0) and also 44 females of *Adlerius* subgenus species (the females of all *Adlerius* species in Iran; their external genitalia provide the only diagnostic morphological characters, the females of remarked species could not be separated morphologically based on the structure of the spermathecae or the weakly developed Pharyngeal armature) in the investigation locations in Sarab province. *Phlebotomus sergenti (Paraphlebotomus)* (total 194: males 99 and females 95), *Phlebotomine papatasi (Phlebotomus*) (total 108: males 56 and females 52) and *Sergentomyia* spp (total 86) were found and identified at this location. The most abundant species was *P. kandelakii*. Details of distribution, fauna, species, sex, dates and habitats of sandflies collected in Sarab district are given in Table 2.

CONCLUSION

Vector incrimination, fauna, distribution and sandfly species' identification of the VL in Sarab district of east Azerbaijan province in north of Iran have not been examined and this is the first report of some sandfly distribution and vector incrimination of VL in this area. However, recently there is a report of infected *P. kandelakii* to *L. infantum* in Khorasan province in border of Turkmenistan[13].

Knowledge of the identification of sandfly populations, ecological aspects, fauna and habitats will be useful for planning interventions in leishmaniasis transmission[14, 15]. Epidemiological studies of leishmaniasis begin with efforts to identify the vector, which is often difficult because there are closely related species that can only be distinguished morphologically in one of the sexes. Out of 700 species of sandflies, 70 have some time been thought to be involved in the transmission of leishmaniasis[16, 17].

Based on abundant of sandflies, we can conclude that *P. kandelakii* is common in this district of new VL focus in east Azerbaijan province. This sandfly may have been acquiring *L. infantum* from dog reservoir hosts living peridomestically[18,19].

A common Haplotype (EU604810) of *L. infantum* was identified in GenBank sequences from strains originating from Sudan and elsewhere in Iran. This haplotype was predominated in Iranian sandflies infected with *L. infantum*[8]. Four female *P. kandelakii* sandflies were found to be infected with *L. infantum*, based on the amplification of fragments of the ITS-rDNA. The widespread distribution of the common Haplotype (EU604810) of *L. infantum* does not suggest a transient introduction, although it could have been introduced in a single host because only one haplotype was unambiguously detected[8, 18].

Infection rate of *L. infantum* among sandflies of the endemic areas is very low[19, 20]. Therefore, it is not surprising that only 4 infected sandflies, have been found in this study. Previously in northwestern Iran not in our study site, *L. infantum* has been reported in *P. kandelakii* and *P. perfilliewi*[7, 8, 13, 20].

According to *P. kandelakii* gonotrophic stage, *Leishmania* infections were found only for females containing large eggs (semi-gravid and gravid) (Table 1). Some of the flies without blood, eggs might have been nulliparous and, therefore, had not the chance to become infected and large quantities of blood might inhibit PCR[11].

By finding the *L. infantum* in *P. kandelakii* for the first time on this new focus, now we can conclude that *P. kandelakii* might be the vector of *L. infantum*. In the future, more works should be done to test the status of *P. kandelakii* as a proven vector of *L. infantum.*

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Conflicts of interest: Not declared.

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