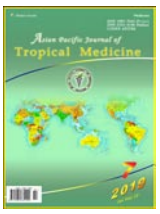

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Visceral leishmaniasis among children in an endemic area of northwestern Iran between 2016 and 2017: An epidemiological study

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ABSTRACT

Objective: To clarify the epidemiological aspects of visceral leishmaniasis in Kaleybar and Khoda-Afarin districts, north-west of Iran.

Methods: A total of 1 420 human (children under 12 years) samples, 101 domestic dogs samples (*Canis familiaris*), and 577 female sand fly samples were collected. Sera of human and dogs were tested using the direct agglutination test, and sand flies were identified at species level using the microscopic method. Furthermore, a structured questionnaire was applied to evaluate the correlation between the potential risk factors and the related clinical signs/symptoms with the human and dogs' seropositivity.

Results: Totally, 2.18% of human samples were positive at titers≥1: 800; among them, 13 cases (41.94%) were above 1:3 200, and clinical symptoms were observed in all of them except for an 11-year old girl. Anti-*Leishmania infantum* antibodies were found at titer≥1: 320 in 9.90% of dogs' samples, half of them had at least one sign of canine visceral leishmaniasis. Moreover, 10 *Phlebotomus* species were identified in the study areas, and *Phlebotomus (Larroussius) major* group was the predominant species. There are significant correlations between the presence of anti-*Leishmania infantum* antibodies and the fever (P<0.001), anemia (P=0.001) and weight loss (P=0.016) in children. On the other hand, significant correlations were revealed between the *Leishmania* infection and the shelter (P=0.039), cutaneous lesion (P=0.005), lymphadenopathy (P=0.001) and weight loss (P<0.001) in the infected dogs.

Conclusions: Visceral *Leishmania* infection is prevalent in rural areas of Kaleybar and Khoda-Afar districts located in East-Azerbaijan province, therefore active detection and treatment of visceral leishmaniasis cases should not be neglected.

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1. Introduction

Leishmaniasis poses a major public health problem worldwide and is caused by protozoan of the genus *Leishmania* spp. More than 20 *Leishmania* species are known as the causative agent of leishmaniasis transmitted by the bites of the infected females of Phlebotomine sand flies[1]. The disease has three main clinical forms including visceral, cutaneous, and muco-cutaneous[1]. Cutaneous leishmaniasis (CL) is more prevalent (between 600 000 to 1 million new cases annually), but the visceral form as a life-threatening disease is more important with 20 000-30 000 deaths annually[1,2].

In Iran, visceral leishmaniasis (VL) has an average of 100-300 cases annually[3,4], showing a decreasing trend due to the improvement of hygienic conditions and living standards[5]. Consequently, most cases have been reported from remote and rural areas, where people have close contact with dogs as the main VL reservoir[6]. Disease caused by *Leishmania* (*L.*) *infantum* affects children more than adults[4,7]. Although the disease has been reported sporadically from different parts of Iran, two endemic foci are more impressive, located in the northwestern (East Azerbaijan and Ardabil provinces) and southern parts (Fars and Bushehr provinces) of Iran[4]. Generally, vectors mostly belong to *Adlerius* and *Larrousius* groups, and the reservoirs to the Canidae family, thus dogs are considered as the main reservoir host in this regard[4,8].

It is noteworthy that, more than half of the infected dogs have no clinical signs, but they can transmit the infection to sand fly vectors, so asymptomatic dogs are as dangerous as symptomatic ones, indicating the necessity for their detection[9]. East Azerbaijan province is regarded as one of the old endemic focus of VL in Iran[10]. Recently, the reported cases from Kaleybar and Khoda-Afarin counties were significant, as 19 cases (13 from Kaleybar and 6 from Khoda-Afrin) were reported during the last 6 years (official report from the health office). To the best of our knowledge, few studies have been performed in these regions. So, there is a great need for an epidemiological study on humans, dogs and sand flies to provide the updated data regarding the recommendations for surveillance and disease control.

Among the common parasitological, serological and molecular methods for VL diagnosis, direct agglutination test (DAT) was used to check the potential seropositivity of humans and dogs sera due

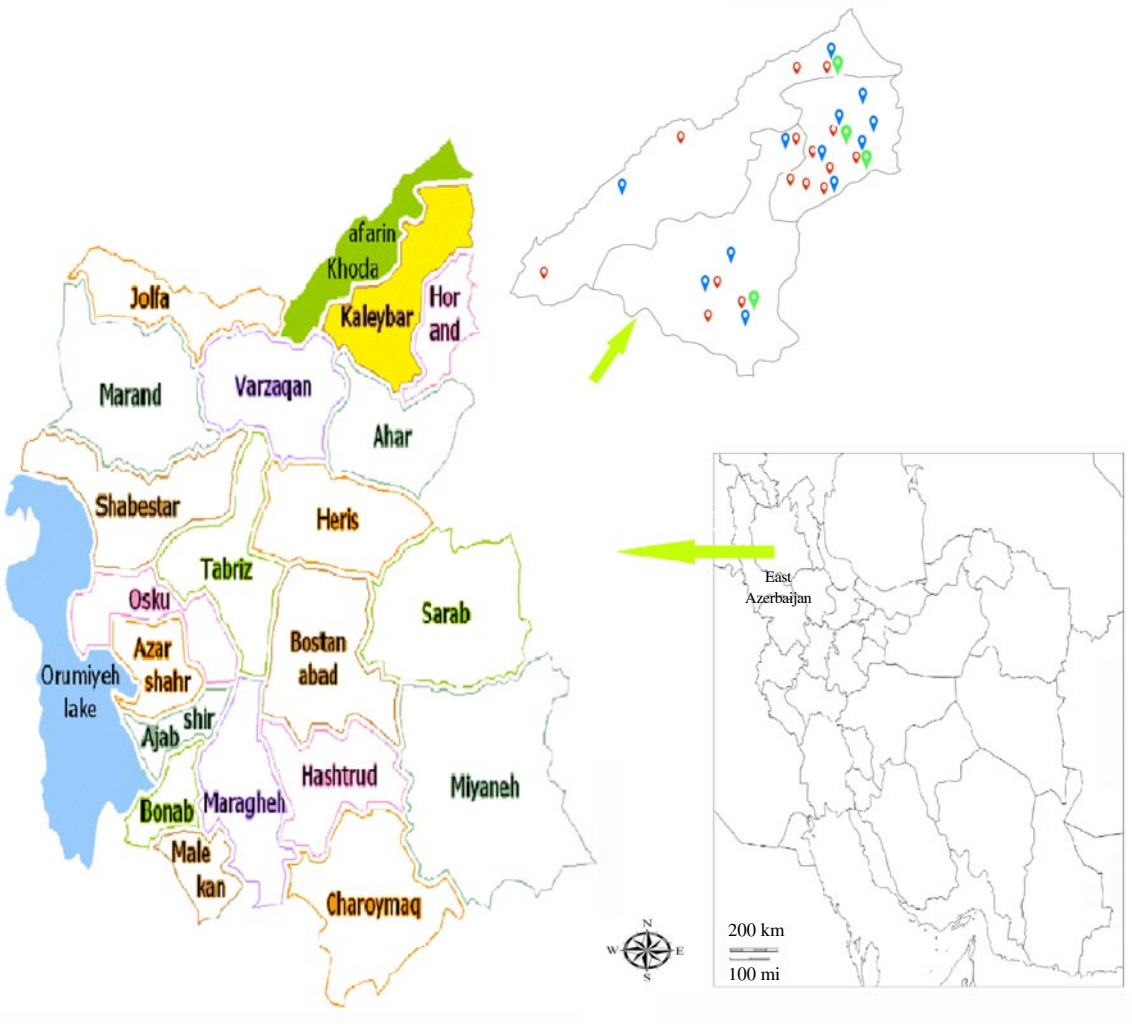

Figure 1. Map of Iran of the study areas and collection sites (Khoda-Afarin and Kaleybar County are marked in green and red, respectively).Green icon: Dog sampling sites; Red icon: Human active sampling sites; Blue icon: Sand flies sampling sites.

to its simplicity, short time of administration, cost-effectiveness, field applicability and more importantly its high accuracy[4]. Also, to study the sand flies fauna, they were collected and were morphologically identified.

2. Material and methods

2.1. Ethics statement

The project was approved by the committee on the ethics of research department, School of Medicine, Shahid Beheshti University of Medical Sciences, under permit number: IR.SBMU. MSP.REC.1395.204 as well as Tehran University of Medical Sciences IR-TUMS. 95-01-162-31832. Written informed consent was collected from all the participants. Permission was obtained from the parents of the children. Enrollment of all dogs in this study was performed with the owner's consent who was informed completely about the procedures and the requirements for follow-up.

2.2. Study areas

Kaleybar and Khoda-Afarin Counties located in East-Azerbaijan province, northwest of Iran (Figure 1) and extend over an area of 3 597 m^2. It is bounded to the north by the Republic of Azerbaijan to the west by Jolfa County to the south by Ahar and Varzaqan Counties and to the east by Ardabil province. The study areas have moderate mountainous weather, with an average altitude of 1 240 m above the sea level. Annual cumulative rainfall is 372 mm, with a mean average temperature of 13.6 ℃.

Base on the 2016 census, the population were 79 120, mostly live in the villages. Animal husbandry comprises the major economies in this area, resulting in more contact with dogs. For sample collections, villages were selected by simple random sampling method.

2.3. Sample collections

We considered three main populations in the studied areas: dogs, humans (children under the age of 12 years old) and sand flies, the details of the collection have been showed in Supplementary Table 1.

2.4. Human subjects

The calculations for size sampling were designed based on the previous reported of VL prevalence in Northwest of Iran (2.9%)[4], so, totally 1 420 human samples were collected and examined. Prior to taking blood samples, a questionnaire was submitted to the participants in order to obtain information about locality, age, gender, style of residency (Nomad or resident), clinical signs, contact with dog, and history of the disease. About 150 microliters blood samples were collected from the finger by pushing a sterile sharp lancet and using 3 regular microhematocrit capillary tubes for each individual, then tubes were sealed. The tubes were placed in a microhematocrit centrifuge and spanned for 3 min at 10 000×*g* for sera separation. All of the sera samples were stored at -20 ℃ until use[11].

2.5. Canine sampling

Considering the previous report on the seroprevalence of dogs in northwestern[12], about 220 blood samples were calculated to be examined which were organized in the same collection sites during September 2017. Only domiciled dogs were entered into this study. Questionnaires including closed questions on the variables associated with the disease were filled out for each dog. During sample collection, dogs were checked for any clinical symptoms consistent with VL and considered to be symptomatic if at least one of the following symptoms was observed: cutaneous lesion, onychomycosis, lymphadenopathy, and/or weight loss (cachexia). These observations alongside with other information such as locality, age, sex, class of dog (sheep, guard, pet dog) and place of living (indoor or outdoor) were recorded. About 3 mL of blood was taken from dogs by cephalic venipuncture using venoject. Sera were separated by centrifugation of blood samples at 800×*g* for 5 min. All sera were then stored at - 20 ℃ until use.

2.6. Vector sampling

Sand flies were collected from July to the late of September 2017 using sticky traps. The sample size was calculated based on the expected infection prevalence 5% reported in a previous study[13], which estimates that 477 sand flies should be collected. Captures were carried out for 15 nights by about 70 sticky traps which were set inside and outsides of houses and domestic animal barns, ruins, grooves of walls and rocks, and rodent nests. Traps were set every day at sunset and were collected the next day after sunrise, and checked each morning. Sandflies were removed by fine needles and brush, washed with acetone, and kept in 70% ethanol at -20 ℃ until use. Then, each female was dissected individually by using microneedles and sterilized forceps. The head and the last few abdominal segments were slide-mounted in Puri's medium, reliable identification keys were used[14-16]. It was done by examining the morphology of female including shape of spermathecae (number of segments, spermathecal duct) and pharynx (pharyngeal teeth and armature), and antennal formula and for male genitalia [aedeagus, coxite, style, spines on style, basal lobe (for *Paraphlebotomus*), length of penis pump, antennal formula].

2.7. DAT

Anti-*L. infantum* antibodies in human and dog samples were detected by DAT. Antigen was prepared in the leishmaniasis Lab of School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. The producer was done based on what stated by other researchers[17]. Serial dilutions to the endpoint of 1: 102 400 for human sera and 1: 20 480 for dog sera were prepared for ones which

found positive in the screening stage. The protocol was similar for human and dog samples with a minor difference, as dog sera were incubated for 60 min at 37 ℃.

After that, 50 microliters (equal volume of serum) of the antigen suspension was added to each well. The combination was incubated for about 18 h and in a humid room at room temperature. Then, the samples were compared with positive and negative controls, and compact blue dot and large diffuse blue mat were considered as negative and positive results, respectively[18]. For human samples, those were considered positive with titer of anti-*Leishmania* antibodies≥1: 3 200, suspected when it was between 1: 800 and 1: 1 600 and negative at titers under 1: 800. Antibody titers≥1: 320 for canines were assumed as positive, between 1: 160 and 1: 80 suspected and negative under 1: 80[6].

2.8. Data analysis

Chi-squared (χ^2) was used to compare seroprevalence values relative to different variables. Analyses were done using IBMI SPSS Statics Software, version 22 (IBMI, NY, USA).

3. Results

3.1. Seroprevalence of VL in human populations

Among 1 420 samples taken from children (aged from 6 months to 12 years old), 31 (2.18%) individuals had anti-*Leishmania* antibodies at titers above 1: 800. Among them, 13 cases (41.94%) were≥1: 3 200, the details are presented in Table 1.

Although the highest seropositivity was observed in the age group under 4 years old (3.38%), no significant correlation was revealed between the seropositive samples and age group as well the gender, residency and dog contact (Table 1). The results of seropositive children (titers≥1:3 200) regarding the collection sites are shown in Table 1. All positive samples were found by active sampling, and just 3 cases were referred to the health center from Shamlou, Lumeh Daraghand, and Ajudan Abad. The majority of positive cases (53.84%) belonged to the Abesh-Ahmad district, Kaleybar County.

Table 1. Visceral leishmaniasis seropositive samples in children blood samples collected from Kaleybar & Khoda- Arfin counties categorized by demographic variables and clinical symptoms [*n* (%)].

Variables	Total	Seropositive samples at different titers			Total	*P*-value
		1:800	1:1 600	1/3 200		
Sex						
Male	708 (49.98)	6 (0.85)	1 (0.14)	8 (1.13)	15 (2.12)	0.868
Female	712 (50.02)	9 (1.26)	2 (0.28)	5 (0.70)	16 (2.25)	
Age (years)						
0-4	562 (39.58)	9 (1.60)	1 (0.18)	9 (1.60)	19 (3.38)	0.082
5-8	492 (34.65)	2 (0.41)	1 (0.20)	2 (0.41)	5 (1.02)	
9-12	366 (25.77)	4 (1.09)	1 (0.27)	2 (0.55)	7 (1.91)	
Residency						
Nomadic	147 (10.35)	0 (00)	0 (0)	2 (1.36)	2 (1.36)	0.476
Resident	1 273 (89.65)	15 (1.18)	3 (0.24)	11 (0.86)	29 (2.28)	
Dog contact						
Yes	995 (70.07)	10 (1.00)	3 (0.30)	11 (1.11)	24 (2.51)	0.999
No	425 (29.93)	5 (1.18)	0 (0)	2 (0.47)	7 (1.65)	
History of fever						
Yes	297 (20.92)	6 (2.02)	1 (0.33)	10 (3.37)	17 (5.72)	<0.001
No	1 123 (79.08)	9 (0.80)	2 (0.18)	3 (0.27)	14 (1.25)	
Hepatosplenomegaly						
Yes	5 (0.35)	0 (0)	0 (00)	5 (100.00)	5 (100.00)	0.999
No	1 415 (99.65)	15 (1.06)	3 (0.21)	8 (0.57)	26 (1.84)	
Anemia						
Yes	465 (32.75)	6 (1.29)	2 (0.43)	11 (2.37)	19 (4.09)	0.001
No	955 (67.25)	9 (0.94)	1 (0.10)	2 (0.21)	12 (1.26)	
Weight loss						
Yes	295 (20.77)	2 (0.68)	1 (0.34)	9 (3.05)	12 (4.07)	0.016
No	1 125 (79.23)	13 (1.16)	2 (0.18)	4 (0.36)	19 (1.69)	

Table 2. Seroprevalence of visceral leishmaniasis in dog blood samples collected from Kaleybar and Khoda-Afarin counties, northwest of Iran 2017 categorized by different titers and site of collections.

County	Villages	No. of examined dogs	No. of seropositive dogs at different titers							Total(%)
			1:80	1:160	1:320	1:640	1:1 280	1:2 560	1:20 480	
Kaleybar	Kalalaq	20	2	1	-	1	-	-	-	4 (20.00)
	Dilbilmaz	30	2	1	-	2	1	1	1	8 (26.67)
	Najaf Tarakameh	20	-	-	-	-	-	-	-	0 (0)
Khoda-Afarin	Ozbak	31	2	4	-	1	1	1	1	10 (32.26)
	Total	101	6	6	-	4	2	2	2	22 (21.78)

Regarding the clinical symptoms, no significant correlation was revealed between the hepatosplenomegaly and the seropositive cases (P=0.999), but it was significantly associated with the fever (P<0.001), anemia (P=0.001) and weight loss (P=0.016). It should be mentioned that no clinical signs were observed for one seropositive case, who was an 11-year old girl with titer=1:3 200. All patients were referred to the physicians for treatment, the follow-up was done after two months for any possible increase in the titer; fortunately, anti-*Leishmania* titers of all patients were decreased, and symptoms were improved.

3.2. Seroprevalence of VL in dogs populations

Out of sera samples of 101 dogs (with mean age of 2.93 years old), 22 (21.78%) of them were seropositive. Anti-*L. infantum* antibodies at a titer of 1:320 and higher were considered as positive, so, only 10 dogs (9.90%) were regarded positive, and mostly were from Dilbilmaz comprising of 5 cases, followed by Ozbak, with 4 cases and just 1 case from Kalalaq (Table 2).

Table 3 shows the results of the seroprevalence analysis in relation to the detailed characteristics of the examined dogs. Most of them were kept as guard and sheepdogs (97.03%), among which 8 (17.78%) seropositive dogs belonged to the sheepdogs, and 9 (16.66%) dogs inhabited outdoors.

Table 3. Seroprevalence of canine *Leishmania infantum* by demographic data and clinical symptoms in dogs collected from Kaleybar and Khoda-Afarin counties, northwest of Iran 2017 [*n*(%)].

Variables	No. of dogs	Seropositive	*P*-value
Sex			
Male	83 (82.17)	10 (12.05)	0.998
Female	18 (17.83)	0 (0)	
Age (years)			
0-3	62 (61.39)	6 (9.68)	0.802
4-7	33 (32.67)	4 (12.12)	
>7	6 (5.94)	0 (0)	
Class			
Pet	3 (2.97)	0 (0)	0.188
Sheepdog	45 (44.55)	8 (17.78)	
Guard dog	53 (52.48)	2 (3.77)	
Shelter			
Outdoor	54 (53.55)	9 (16.67)	0.039
Indoor	47 (46.45)	1 (2.13)	
Cutaneous lesion			
Yes	6 (5.94)	3 (50.00)	0.005
No	95 (94.06)	3 (31.58)	
Onychomycosis			
Yes	4 (3.96)	4 (100.00)	0.999
No	97 (96.04)	0 (0)	
Lymphadenopathy			
Yes	5 (4.95)	5 (100.00)	0.001
No	96 (95.05)	1 (1.04)	
Weight loss			
Yes	9 (8.91)	5 (5.43)	<0.001
No	92 (91.09)	4 (44.44)	

As shown in Table 3, the highest seroprevalence rate (12.2%) was observed among the domestic dogs aged between 4-7 years old, and no seropositive dog was detected at the age of above 7 years old. No seropositive dogs were found among females, but the difference was not statistically significant (P=0.998).

At least one of the clinical signs (cutaneous lesions, onychomycosis, lymphadenopathy and weight loss) was observed in half of the seropositive dogs (at titers of≥1: 320) (Table 4). The seropositivity of *Leishmania* infection was significantly associated with the shelter (P=0.039) cutaneous lesion (P=0.005), lymphadenopathy (P=0.001) and weight loss (P<0.001) (Table 3).

3.3. Identification of Phlebotominae sand flies

A total of 577 female sand fly samples were captured, belonging to the two genera of *Phlebotomus* (277) and *Sergentomyia* (300). According to the objectives of the current study, the *Sergentomyia* spp. was just identified at genus level, but the *Phlebotomus* samples were identified at species level. Overall, 10 species were identified in the *Phlebotomus* genus (Table 5).

Table 6 summarizes the results of the *Phlebotomus* identification categorized by the studied areas considering the total seropositive samples in dogs and human cases.

Table 4. Distribution of symptomatic and asymptomatic dogs by seroprevalence rate of *Leishmania infantum* in Kaleybar and Khoda-Afarin counties, East Azerbaijan, northwest Iran [*n*(%)].

Result of DAT test	No of dogs	Symptomatic	Asymptomatic
Titer≥320	10 (9.90%)	5 (50.00%)	5 (50.00%)
Titer<320	12 (11.88%)	2 (16.67%)	10 (83.33%)
Negative*	79 (78.22%)	4 (5.06%)	75 (94.94%)
Total	101 (100.00)	11 (10.89%)	90 (89.11%)

*No anti -*Leishmania* detected.

Table 5. Diversity of sandflies in Kaleybar and Khoda-Afarin counties, East Azerbaijan, northwest Iran.

Species	Number
Female	126
Major group	
Adlerius group	50
Phlebotomus (Phlebotomus) papatasi	46
Phlebotomus (Paraphlebotomus) sergenti	38
Phlebotomus (Larrousius) kandelaki	7
Phlebotomus (Paraphlebotomus) caucasicus group	7
Phlebotomus (Paraphlebotomus) alexandri	2
Unknown	1
Male	
Phlebotomus (Larroussius) perfiliewi	105
Phlebotomus (Paraphlebotomus) sergenti	40
Phlebotomus (Larroussius) neglectus	30
Phlebotomus (Adlerius) halepensis	20
Phlebotomus (Phlebotomus) papatasi	8
Phlebotomus (Larrousius) kandelaki	3
Phlebotomus (Larrousius) tobbi	3
Phlebotomus (Adlerius) simici	1
Phlebotomus (Paraphlebotomus) alexandri	1
Phlebotomus (Paraphlebotomus) caucasicus	1
Unknown	10

4. Discussion

Control of VL as a neglected and emerging disease is mainly focused on the early diagnosis and treatment of human cases, surveillance, monitoring and control of vectors and reservoirs, followed by the environmental management, and health education, which all need an updated and reliable data[2,4]. Therefore, the current study was aimed to provide such data on three main elements of disease making up the chain of transmission (human, dogs, sand flies).

Although Kaleybar and Khoda-Afarin counties in East Azerbaijan province, Iran, are considered as old endemic foci, there are limited available data. Here, updated epidemiological information on human, dog and sand flies as vectors was achieved, hoping to provide useful and basic data for disease control.

VL has been reported to affect mainly the children, especially in the endemic areas of Mediterranean basin[4], so in the current study, all the examined cases belonged to the children. In Iran, the VL prevalence ranges from 0.07% to 10.15% depending on the region and the used diagnostic method[4]. The present study showed a positivity of 2.8%. The dominant cases were found in the age group of 0-4, but it was not statistically significant. All children aged younger than one year old were found to be seronegative, corroborating with some other researches, which could be due to their complete coverage and staying mostly indoors, resulting in lesser contact with vectors, as well as the long latency of VL[4]. No significant relation was found for other variables such as dog contact, gender, or type of residency.

Leishmania infection can cause a wide range of clinical manifestations varying from asymptomatic to fatal form, but asymptomatic form of *Leishmania* infection is one of the most reported forms in Iran and other countries such as India, Turkey, Croatia, and Brazil[17,19-21]. In Iran, paleness is the main reported clinical sign of VL[4]. The results showed no clinical signs in seropositive cases with antibody titer below than 1: 1 600. Except for an 11-year old girl, all of other children showed at least one clinical sign at a titer of 1: 3 200. The results of analysis showed a significant correlation between the presence of anti-*Leishmania* antibody and all the observed clinical signs expect hepatosplenomegaly. Most of the seropositive cases had the titers ranging from 1: 800 to 1: 1 600, and did not have any marked symptoms, showing that the cases were detected very early.

Results highlighted that most of the present seropositive cases belonged to Abesh-Ahmad district similar to the previously reported

Table 6. Phlebotomus species collected from Kaleybar and Khoda-Afarin counties, northwest of Iran 2017 considering the positive anti-*Leishmania* antibody cases of humans and dogs.

Collection site	Sero$^+$ human [*n*(%)]	Sero$^+$ dog [*n*(%)]	Collected sand flies	
			Female species	Male species
Kaleybar				
Kalalaq	2 (2.53)	1 (5.00)	*Major* group, *Adlerius* group, *Phlebotomus kandelaki*	*Phlebotomus sergenti, Phlebotomus simici, Phlebotomus kandelaki, Phlebotomus perfiliewi, Phlebotomus neglectus* and *Phlebotomus halepensis*
Dilbilmaz	5 (3.73)	5 (16.60)	*Major* group, *Adlerius* group, *Phlebotomus caucasicus group, Phlebotomus papatasi, Phlebotomus kandelaki, Phlebotomus sergenti*	*Phlebotomus sergenti, Phlebotomus perfiliewi, Phlebotomus neglectus*
Ghayebashi	5 (2.72)	-	*Major* group, *Adlerius* group, *Phlebotomus sergenti, Phlebotomus papatasi*	*Phlebotomus perfiliewi, Phlebotomus neglectus, Phlebotomus papatasi, Phlebotomus sergenti, Phlebotomus caucasicus, Phlebotomus halepensis*
Najaf Tarakameh	1 (1.09)	0	*Major* group, *Adlerius* group, *Phlebotomus alexandri, Phlebotomus papatasi, Phlebotomus sergenti*	*Phlebotomus perfiliewi, Phlebotomus kandelaki, Phlebotomus alexandri, Phlebotomus halepensis, Phlebotomus sergenti, Phlebotomus tobbi, Phlebotomus neglectus*
Oti Kandi	1 (1.05)	-	*Adlerius* group	*Phlebotomus perfiliewi, Phlebotomus halepensis*
Pirelare ShahGasem	2 (11.11)	-	*Adlerius* group, *Phlebotomus kandelaki* and *Phlebotomus sergenti*	*Phlebotomus sergenti*
Gale Kandi	4 (2.20)	-	-	-
Kaleybar	3 (2.80)	-	*Major* group	Not caught
Lumeh daragah*	1	-	-	-
Ajudan Abad*	1	-	-	-
Shamlou*	1	-	-	-
Khoda-Afarin				
Ozbak	2 (1.47)	4 (12.90)	*Major* group, *Adlerius* group, *Phlebotomus sergenti, Phlebotomus papatasi*	*Phlebotomus perfiliewi, Phlebotomus caucasicus, Phlebotomus papatasi, Phlebotomus halepensis, Phlebotomus sergenti, Phlebotomus neglectus*
Aliverdi Ushaghi	3 (2.75)	-	-	-

*Thses 3 cases were referred to the health center by physicians not by active detction.

VL patients in recent years. Most of the residents were found to live in the rural environment with more dog contact, poor condition of housing, lack of sanitation, low income, accumulation of garbage around the houses, more outdoor activities and being nearer to the breeding sites of domestic animals. The latter could also lead to the increase in the vector breeding areas by providing enough amounts of organic materials. This area has also a lower altitude and slightly higher temperature, which could provide more appropriate condition for the vectors.

Dogs are the main reservoir of the disease in the zoonotic transmission cycle[22]. Previous studies have not reported any cure for canine visceral leishmaniasis yet, so infected animals should be immediately detected and quarantined. In most cases, infection is asymptomatic or the clinical signs of the infected dogs are nonspecific and variable, therefore diagnosis and management would be difficult. Infected dogs should be detected by the parasitological and/or serological methods like DAT[22]. It should be mentioned that the total number of the tested canine blood samples in the current study were less than the estimated one based on the sample size formula, resulting from the lack of budget and cooperation of dogs' owners, leading to a final sample size of 101 cases. The results showed a seropositivity of 21.78% among the examined dogs. In Iran, the seroprevalence rate varies from 5.4% to 19.6%[4], due to the different classes of dogs, sampling methods, and the area specifications, with the highest amount reported (19.6%) in the northwest, Meshkin-Shahr County[4]. The cases of seropositive dogs were notified in areas the same as the human cases in both counties (Dilbilmaz and Ozbak), indicating the role of canine in the disease cycle. The results showed that the seropositive cases are much higher in dogs inhabiting outdoors and sheepdogs, reflecting their more exposure to the vector bites.

The main defined clinical symptoms of the infected dogs are cachexia, hair loss, cutaneous lesions, weight loss, lymphadenopathy and lethargy[23], but most of the time the infected dogs are asymptomatic[4,23]. The results of current study showed that at least one of the following clinical signs (cutaneous lesions, onychomycosis, lymphadenopathy, and weight loss) was observed in half of the seropositive dogs with a titer of $\geqslant$1: 320, but 50% of them were asymptomatic, showing their exposure to the infection without any apparent clinical signs. It is consistent with the results of other studies[23-25], highlighting the fact that not only their role has not been mitigated in disease transmission, but also it may worsen the situation due to being neglected in the elimination programs. It could be concluded that further studies are needed with more sample size, especially on stray dogs to provide the exact prevalence of dogs' infection in order to manage the control programs and any possible prediction of disease re-emergence.

Fanustic study on the Phlebotominae sandflies was another aspect of the current study. All the identified species were among the Iranian sand flies fauna previously reported in the study areas[26].

Among the known vectors of canine visceral leishmaniasis in Iran, *Phlebotomus* (*Phlebotomus*) *alexandri* was found in very few cases from Najaf Tarakameh. This species was considered as a vector of VL in the southern part of Iran, Khuzestan and Fars Counties[26,27], but its role should be investigated more in the transmission of disease in the north-western part of the country.

Few numbers of *Phlebotomus caucasicus* group was caught during this study, the first record of promastigote infection of *Phlebotomus caucasicus* in a VL focus was reported in the northwest of Iran (Ahar) in 2004, but its contribution to VL transmission needs to be investigated more[28].

Sandflies belonging to the subgenera of *Larroussius* and *Adlerius* are known as primary vectors of zoonotic visceral leishmaniasis in the world as well as Iran[13,29]. The medical importance of *Adlerius* subgenus has been unknown for a long time, Killick-Kendrick introduced some of the *Adlerius* species as possible vectors of VL[30], but later other researchers proposed the role of the members of this subgenus in the transmission of VL and CL[31-33]. The *Adlerius* species is identified just based on the male morphology, and females cannot be distinguished at species level, they just could be identified at subgenus level based on the wrinkled spermathecae with irregular segments, a short neck with gradually reduced width toward the end[34-36]. In some selected sites, where human and dogs' seropositive cases were found or/and frequency of *Adlerius* samples was high, species for male samples of *Adlerius* group were also identified, expanding our view on the fauna. In the most collection sites, *Adlerius* members were captured, based on the morphological characteristics all male species were identified as *Phlebotomus halepensis*, roughly it could be figured out that the female species also were *Phlebotomus halepensis*. This species is the most frequent species of *Adlerius* subgenus in Iran[34], and was previously found to be infected to *Leishmania* in Meshgin-Shahr[37].

At least 12 species of the subgenus *Larroussius* are the proven or probable vectors of leishmaniasis[30]. In the current study, among female samples, eudominant species was the member of this subgenus accounting for 48.01% of samples in the total collection. The subgenus is identified by a long extension of the spermathecal neck in female species, but similar to *Adlerius* group, determination of some female species of *Larroussius* is impossible based on the common morphological characteristics[38,39]. It could make complication for vector incrimination in zoonotic visceral leishmaniasis foci, where these species found sympatrically such as northwestern of Iran. But, according to some researchers, it is possible to differentiate them based on the base of spermathecal ducts[15,30,40]. This characteristic is reliable but is not easily found in mounted samples, especially when they are not properly dissected or in gravid or sub-gravid samples. Herein, the failure to identify the female species based on the shape of spermathecal ducts could be due to the mentioned reasons. Since the identification of *Larroussius* subgenus in male species is easier based on the genital terminalia, in this study the male species composition was detected in order to guess roughly that of female species. In all

sites where female species of *Major* group were found, *Phlebotomus neglectus*, *Phlebotomus tobbi* and *Phlebotomus perfiliewi* (male) were also detected. All three species are among known VL vectors in Iran[26,41].

Unlike the mentioned species, *Phlebotomus* (Larr.) *kandelaki* could be easily distinguished in female species based on the number of spermathcal segments[14,15]. This species was found in 3 collection sites (Kalalagh, Pirelare and Dilbilmaz). Natural infection of this species to *L. infantum* was recorded in Meshgin-Shahr, northwest of the country[26,42].

During the current study, interestingly, *Larrossius* group was caught in the most human and dog seropositive areas, except in few villages which may be due to the small sample size of the collected samples, implying their role in the VL transmission. Further studies on the vectorial capacity and detection of *Leishmania* species in collected sandflies, humans and dogs are recommended undergoing by the same authors.

An updated epidemiological study is a crucial factor for any planning regarding the control strategies. In the present study, most of the seropositive children belonged to the Abesh-Ahmad district, implying that these areas have more suitable conditions for *Leishmania* transmission, where farming, livestock, and domestic and wild dogs are more prevalent than the other parts of the province. East Azerbaijan province is one of the areas with more migrants and tourists due to its appropriate climate. VL infection in the province underlines the need for health and therapeutic achievements for prevention, diagnosis, and treatment of this disease. Furthermore, it is necessary to increase the awareness and alertness among the physicians and public health managers, particularly in high-risk rural parts of the studied areas. More importantly, most of VL cases become fatal if left untreated, but in the present study, using the DAT led to the successful identification of 12 children with clinical features of kala-azar. In conclusion, the findings of our study indicated that the visceral *Leishmania* infection is prevalent in rural areas of Kaleybar and Khoda-Afar districts located in East-Azerbaijan province, northwest of Iran and DAT could be considered in the health policy-making of the area for more precise detection and treatment of VL cases.

Conflict of interest statement

We declare that we have no confilct of interest.

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