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Original Article

An Eco-Epidemiological Study on Zoonotic Cutaneous Leishmaniasis in Central Iran

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Abstract

Background: Leishmaniasis is an expanding neglected tropical disease in the world reporting from 98 countries including Iran. This study focused on eco-epidemiological determinants of the disease following a rapid and unexpected increase of leishmaniasis incidence in a strategic residential district in North-East of Isfahan County, Iran.

Methods: This study was accomplished from Apr 2012 to Jan 2014 in a strategic residential zone in North-East of Isfahan County, Esfahan, Iran. Monthly activity, parity, *Leishmania* infection and susceptibility tests, were determined on sand flies. Some portion of inhabitants and school children were surveyed to find active or passive cases of leishmaniasis and also wild rodents were collected to determine reservoir host.

Results: Totally 5223 sand flies belonging to *Phlebotomus* and *Sergentomyia* genus were collected and identified; *Ph. papatasi* was the dominant species and started to appear in May and disappeared in Oct. The majority of living dissected sand flies were unfed and parous. *Ph. papatasi* showed 4.6% *Leishmania* infection through direct examination and 39.54% by nested-PCR respectively. *Phlebotomus papatasi* was susceptible against deltametrin 0.05%. Totally 2149 people were surveyed and incidence and prevalence of zoonotic cutaneous leishmaniasis estimated as 45.39 and 314.40 per 1000 population. Rodents showed 73.91% and 80% *Leishmania* infection by direct examination and nested-PCR respectively.

Conclusion: Cutaneous leishmaniasis due to *L. major* has been established in this area. Rodent control operation and personal protection are highly recommended to control the disease in this focus.

Keywords: Eco-epidemiological; Zoonotic cutaneous leishmaniasis; Host; Vector; Iran

Introduction

Leishmaniasis is an expanding neglected tropical disease in the world reporting in 98 countries including Iran. More than 350 million people are at risk and 12 million are suffering from the diseases and its side effects (1,2). Zoonotic cutaneous leishmaniasis (ZCL) has been reported from 17 out of

31 provinces in Iran (3) and *Phlebotomus papatasi* is considered as the main vector of the disease (4). *Rhombymos opimus* (great gerbil) and *Meriones libycus* (Libiyan jird) are considered as the main and secondary reservoir hosts of the disease in the northeast and central part of Iran respectively (5).

In the recent years eco-epidemiological changes such as urbanization, constructions of buildings near colonies of the rodents, expanding rodent population due to human intervention in their natural habitats and immigration of non-immune people to the endemic areas, caused a significant distribution of ZCL in the country specially in Isfahan (6). Lack of sufficient information about different reservoir host, sand fly vectors, insecticide resistance among the vectors and resistance to the conventional drug/s and also inaccessibility to a licensed vaccine made the control of disease challenging (7).

It is postulated that, successful establishment of the disease in an endemic area is the outcome of a close association between the *Leishmania* parasite/s and its natural sand fly vector/s (8). Thus, vector and parasite identification has a great impact on predicting expansion of the disease in the endemic areas, and also it helps authorities to design new strategic control program of the disease (9).

Following a rapid and unexpected increase in the incidence of ZCL in a residential strategic area in north east of Isfahan City (personal communication, Isfahan Health Center, Iran), conducting a survey on different eco-epidemiological aspect of the disease was absolutely essential and encouraged the authors to study eco-epidemiological determinants of the disease in the area for proposing an integrated control program. This is the first comprehensive study in this high risk strategic focus.

Materials and Methods

Ethics approval

All experiments were approved by Research Deputy of Tehran University of Medical Sciences (92-02-27-23198).

Study area

This study was accomplished from Apr 2012 to Jan 2014 in a strategic residential zone in North-East of Isfahan County (51°51´17"E and 32°44´49"N) with the elevation of 1534.5 m, Iran. The average annual minimum and maximum temperature recorded as 4.7 °C and 36.3 °C in Jan and July, respectively. The total annual rainfall was 157.5 mm. (Iran Meteorological Organization, 2012).

Entomological survey

Sand fly collection and identification

Sand flies were collected from indoors (bedroom, bathroom, toilets and hall) and outdoors fixed places (rodent burrows) using 30 sticky traps sunset to sunrise.

The head and last two abdominal segments of the sand flies were mounted in puris' medium (10) and identified using valid identification keys (11,12).

Living sand flies were collected using mouth aspirator from outdoor on a car parked near gerbils' colonies.

Physiological status and parity test

Abdominal status (unfed, fresh fed, semi-gravid and gravid) of sand flies were recorded. Parous females were distinguished from nulliparous sand flies by observation of the follicular relies of the ovaries (13), and also the appearance of the accessory glands and the genital atrium (14).

Leishmania infection

Female sand flies, captured from rodent burrows, were dissected in a fresh drop of sterile saline (9/1000) and checked for the presence of promastigote form of parasite inside the alimentary canal in Aug and Sept 2013. Samples were subjected to molecular experiment to further confirmation.

Susceptibility tests

Adults were left undisturbed at least for one h, and then the susceptibility tests were carried out according to the WHO recommendation, modified for sand flies by Saeidi et al (7). Sand flies were transferred into the exposure tubes at pre-defined

time intervals, and then mortality was recorded after 24 h in optimum condition; cases of 5 to 20 percent mortality, were corrected by Abbott's formula (15).

Population survey

During 2012, randomly 300 households were questioned to gain data such as: age, sex and the presence of acute lesions or scars of leishmaniasis once a season. Additionally, 1184 students from 7 schools, 7-18 yr. old, were questioned.
Microscopic slides from active lesion/s were prepared. The lesion serosity was transferred on the slides as well as into alcohol 96% for complementary molecular parasite detection.

Reservoir survey

From Apr 2012 to Mar 2013 to determine the reservoir host/s and detection of *Leishmania* infection, rodents were collected monthly using at least 20 Sherman traps, baited with cucumber and/or bread near gerbils' colonies. Impression smears were prepared from the rodents' ears, stained by Giemsa and were observed microscopically for *Leishmania* amastigote form. Finally, both ears were cut and transferred into a 1.5 ml microtube to conducting supplementary molecular experiments.

Molecular study

DNA extraction

Genomic DNA from sand flies, human and rodent samples was extracted using Exgene™ Tissue SV (GeneAll®, Korea), following the manufacturers protocol with some modifications.

Polymerase chain reaction

Ribosomal Internal Transcribed Spacer 2 region (ITS 2) of *Leishmania* parasite DNA was amplified by nested-PCR in an Applied Biosystems thermocycler using external and internal primers; Leish out F (5'-AAA CTC CTC CTC GGT GCT TGC – 3') and Leish out R (5'-AAA CAA AGG TTG TCA GGG G-3') Leish in F (Forward: 5'-AAT TCA ACT TCG CGT TGG CC-3') and Leish in R (Reverse: 5'-CCT CTC TTT TTT CTC TGT GC-3'). The PCRs were carried out based on previous study (16) with minor modification.

Restriction Fragment Length Polymorphism

To distinguish common *Leishmania* species cycling among vector/s reservoir/s and human in the region, amplified DNAs were digested by Rapid Digest Mnl1 (Cat. No: RD 1191) according to the company's instruction. The products were loaded onto 2.5% (w/v) agarose gel electrophoresis in Tris/Borate/EDTA (TBE) buffer. Finally, gels were stained by ethidiumbromide (0.5 µg/ml), and photographed.

Statistical analysis

Data were analyzed using SPSS (Chicago, IL, USA) version 18 and Fisher's exact tests were used to compare incidence and/or prevalence differences between male and female or ages.

Results

Entomological survey

Totally 5223 sand flies were collected and identified from the studied area (Table 1). *Phlebotomus papatasi* was the dominant species in both indoor and outdoor places; reached to its highest activity in early Jul and early Aug in outdoors and indoors, respectively (Fig. 1). The sex ratios calculate at 72.70 in outdoors and 137.5 in indoors for *Ph. papatasi*.

Physiological status, parity and natural Leishmania infection

In 2012, as much as 69 samples of sand flies were captured by sticky traps and the majority was identified as un-fed and parous. In addition, at the following year (2013), 101 living sand flies were captured by aspiration and all of them were identified as *Ph. papatasi*. The majority of the collected sand flies were unfed and parous (Table 2). Among 130 female *Ph. papatasi*, checked microscopically, 6 (4.6%) had natural *Leishmania* infection, and no infection was detected in other species (Table 2). The result of nested-PCR revealed *Leishmania* infection in 5 *Ph. papatasi* (17.85%) (Table 3).

Table 1: The fauna, the number and percentages of sand flies from indoors and outdoors in the studied area, North-East of Isfahan city, Isfahan Province, Iran 2012

Species	***Outdoor***				***Indoor***				***Total***	
	Male		**Female**		**Male**		**Female**			
	No.	%	No.	%	No.	%	No.	%	No.	%.
Ph. papatasi	1137	42.1	1564	57.90	22	57. 9	16	42.1	2739	52.44
Ph. ansarii	610	42.07	840	57.93	1	33.33	2	66.67	1453	27.81
Ph. caucasicus	149	46.41	172	53.59	0	0	0	0	321	6.14
Ph. sergenti	0	0	239	100	0	0	0	0	239	4.57
Se. sintoni	55	11.73	414	88.27	1	50	1	50	471	9
Total	1951	37.66	3229	62.34	24	55.81	19	44.19	5223	100

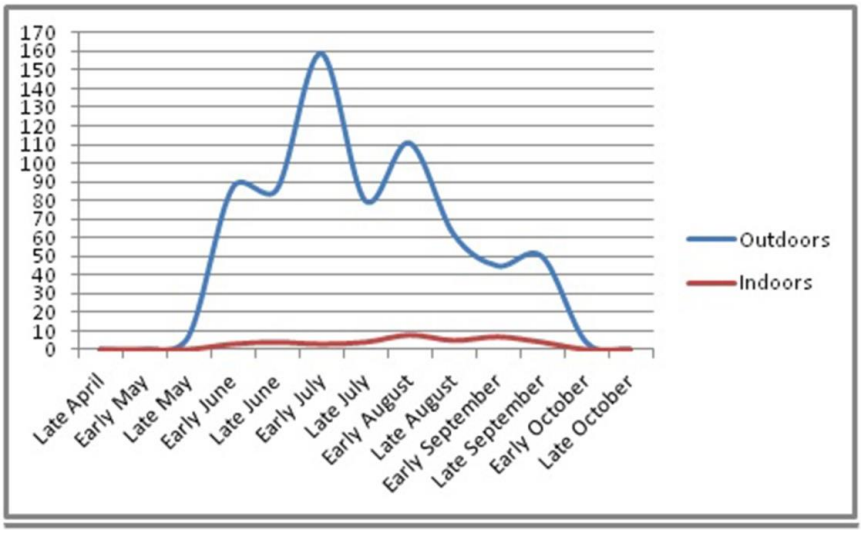

Fig. 1: Seasonal activity of *Phlebotomus papatasi,* in the studied area, North-East of Isfahan city, Isfahan Province, Iran 2012

Table 2: Physiological status, parity and *Leishmania* infection rate of sand flies, collected form rodent burrows, North-East of Isfahan City, Isfahan Province, Iran 2012-2013

Year/collecting method	***Species***	***Physiological status***				***Parity***			***No. infected***	***Infection Rate***	***Total***
		Un-Fed	**Blood Fed**	**Gravid**	**Semi-gravid**	**Parous**	**Nulli-parous**	**Unrecog-nized**			
2013/ Mouth aspirator	*Ph. papatasi*	97	0	1	3	74	27	0	5	4.95	101
2012/Sticky trap	*Ph. papatasi*	16	6	2	5	22	5	2	1	3.44	29
	Ph. ansari	10	7	1	3	16	4	1	0	0	21
	Ph. caucasicus Group	5	1	2	1	6	2	1	0	0	9
	Ph. sergenti	4	1	3	0	7	1	0	0	0	8
	Se. sintoni	1	1	0	0	2	0	0	0	0	2
Total		133	16	9	12	127	39	4	6	3.53	170

Among 101 live *Ph. papatasi* specimens, the parasite infection observed in 5 (4, 95%) under stereomicroscope (Table 2, Fig. 2), though nested-PCR showed 45.55% *Leishmania* infection (Table 3 and Fig. 3). Additionally, out of 129 *Ph. papatasi*, subjected for PCR, 10 were infected by *L. turanica* and 5 had mixed infection of *L. major* and *L. turanica* (Table 3, Fig. 3 and Fig. 4). Among 3 tested *Ph. ansari,* one was found infected by *L. turanica* (Table 3). Visually distinguished sequence of detected *Leishmania* species, digested by mnl1, shows in Fig. 5.

Table 3: Results of molecular *Leishmania* detection in sand flies captured in North-East of Isfahan City, Isfahan Province, Iran 2012-2013

Year/collecting method	*Species*	*No.*	*L. major*		*L. turanica*		*Mix(L. major &L. turanica)*		*Total infection*	
			No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
2012/Sticky trap	*Ph. papatasi*	28	4	14.28	0	0	1	3.57	5	17.85
	Ph. sergenti	1	0	0	0	0	0	0	0	0
	Ph. ansarii	3	0	0	1	33.33	0	0	1	33.33
2013/Aspirator	*Ph. papatasi*	101	32	31.68	10	9.9	4	3.96	46	45.55
Total		133	36	27	11	8.27	5	3.75	52	39.1

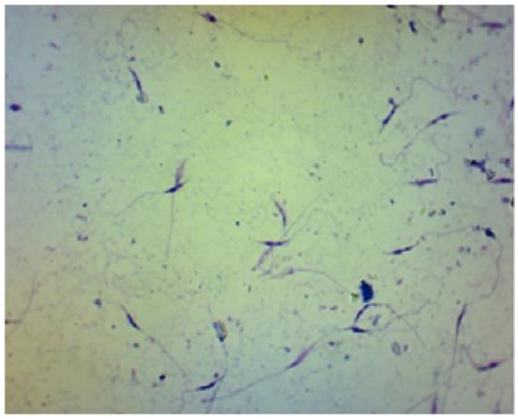

Fig. 2: *Leishmania major* promastigote form, detected from an infected *Phlebotomus papatasi* digestive tract, stained by Geimsa

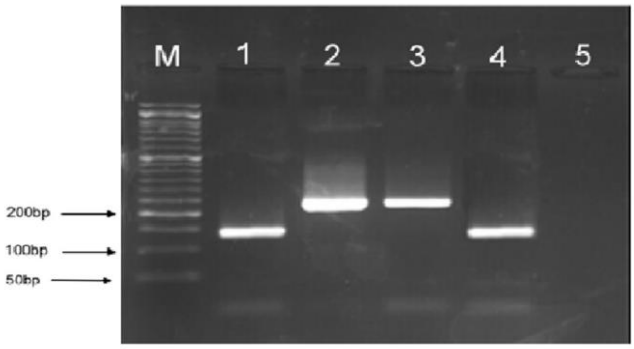

Fig. 3: Nested-PCR amplification of DNA extracted from *Phlebotomus papatasi*, M, 50 bp ladder (Fermentas); lane 1, *Leishmania turanica*; lane 2, *L. major*; lane 3, *L. major* reference strain (MRHO/IR/75/ER); lane 4, *L. turanica* reference strain (MRHO/SU/1983/MARZ-051); lane 5, negative control (distilled water)

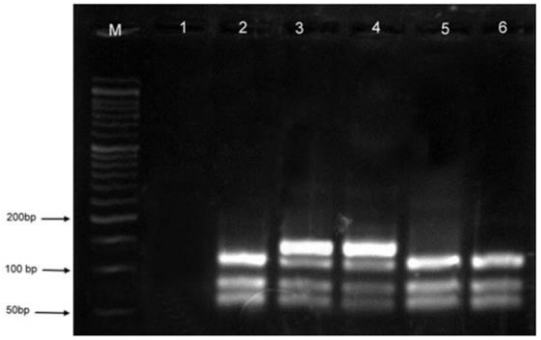

Fig. 4: Agarose (2%) gel electrophoresis of nested PCR products digested by MnlI restriction enzyme, M, 50bp ladder (Fermentas): lane1, negative control (distilled water); lane 2, 5 and 6, *L. major*; lane3 and 4 mixed infection of *L. major* and *L. turanica*.

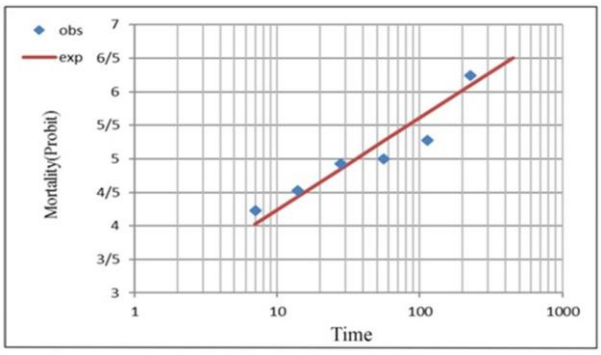

Fig. 5: Probit regression lines of Deltamethrin 0.05% against *Phlebotomus papatasi* using WHO modified protocol

Susceptibility tests

Totally, 519 adult *Ph. papatasi* were subjected to susceptibility tests against Deltametrin 0.05%, in 22 replicates. The mortality rates against Deltametrin 0. 05% for 7, 14, 28, 56, 113, 225, and 450 sec of exposure time, passing 24 h recovery, were 12.11, 23.13, 40.45, 43.63, 55.97, 87.56 and 100 % respectively. The parameters of regression line to Deltametrin 0.05%, values of LT_{50} 95% and LT_{90} 95% to Deltametrin 0.05% are shown in Table 4. The Probit regression lines are shown in Fig. 5.

Human infection

Among 965 inhabitants 31 persons (58.06% male and 41.94% female) had active lesion of cutaneous leishmaniasis. Maximum and minimum of active lesions were seen in ≥ 25 yr. old and 20-25 yr. old age groups. Out of 965 surveyed people 299 persons (51.5 % male and 48.5% Female) had scar. In 2012 incidence and prevalence of ZCL estimated as 46.55 and 341.97 per 1000 population, respectively (Table 5). Furthermore 1184 students were questioned and examined for leishmaniasis, 26.52% and 1.77% of the students had scar and active lesion respectively (Table 6).

Reservoir host examination

Totally 58.62% and 80% of examined rodents were infected by *Leishmania* parasite using direct examination and nested-PCR respectively (Table 7).

Table 4: Parameters of probit regression lines of Deltametrin 0.05% against *Phlebotomus papatasi*

Y=a+bx	*P-value (Heterogeneity)*	*χ2 (df)*	*LT50 95%CI[d]*	*LT90 95%CI[c]*	*B±SE[b]*	*A[a]*
Y = -2.1252 +1.3677 X	0.05<	18.259(5)	62.8821 35.7989 17.3820	1472.1250 309.6956 149.7231	1.3677±0.230	-2.1252

[a] *A* = intercept.
[b] *B*± SE = slope and its standard error.
[c] LT50, 95% CI = lethal time causing 50% mortality and its 95% confidence interval.
[d] LT90, 95% CI = lethal time causing 90% mortality and its 95% confidence interval.

Table 5: The prevalence of active lesions and scar rate by seasons and district, North-East of Isfahan City, Isfahan Province, Iran 2012

	Spring				*Summer*				*Fall*				*Winter*				*Incidence (Per 1000)*
District	Population	Scar/s	Active	Freq. Active (%)	Population	Scar/s	Active	Freq. Active (%)	Population	Scar/s	Active	Freq. Active (%)	Population	Scar/s	Active	Freq. Active (%)	
1	349	78	0	0	350	78	3	11.03	357	81	6	21.74	359	87	0	0	33.09
2	273	75	0	0	275	75	5	25	277	80	4	20.29	280	84	0	0	45
3	323	115	0	0	326	115	8	37.91	323	123	5	25	326	128	0	0	61.61
Total	945	268	0	0	951	268	16	23.42	957	284	15	22.29	965	299	0	0	46.55

Table 6: The prevalence of active lesions and scar rate by age among the students (both sexes), North-East of Isfahan City, Isfahan Province, Iran, 2012

Genus	***Male***					***Female***					***Total***				
Age	**No. examined**	**No. scars**	**Scar (%)**	**No. Active**	**Active (%)**	**No. examined**	**No. scars**	**Scar (%)**	**No. Active**	**Active (%)**	**No. examined**	**No. scars**	**Scar (%)**	**No. Active**	**Active (%)**
7	103	21	20.38	2	1.94	121	17	14.04	1	0.82	224	38	16.96	3	1.33
8	65	16	24.61	4	6.15	94	22	23.40	2	2.1	159	38	23.89	6	3.77
9	68	25	36.76	2	2.94	81	19	23.45	2	2.46	149	44	29.53	4	2.68
10	61	19	31.14	0	0	62	12	19.35	1	1.61	123	31	25.20	1	0.81
11	88	16	18.18	0	0	91	31	34.06	2	2.19	179	47	26.25	2	1.11
12	51	20	39.21	0	0	9	9	100	0	0	60	29	48.33	0	0
13	29	13	44.82	1	3.45	69	21	30.43	2	2.89	98	34	34.69	3	3.06
14	61	16	26.22	1	1.64	47	20	42.55	1	2.12	108	36	33.33	2	1.85
15	18	2	11.11	0	0	0	0	0	0	0	18	2	11.11	0	0
16	30	8	26.66	0	0	0	0	0	0	0	30	8	26.66	0	0
17	33	4	12.12	0	0	0	0	0	0	0	33	4	12.12	0	0
18	3	3	100	0	0	0	0	0	0	0	3	3	100	0	0
Total	610	163	26.72	10	1.63	574	151	26.30	11	1.91	1184	314	26.52	21	1.77

Table 7: Number of collected rodent and their infectivity to *Leishmania*, North-East of Isfahan city, Isfahan Province, Iran 2012-2013

Species	***No. Male (%)***	***No. Female (%)***	***Total***	***Positive by direct examination (%)***	***Examined/positive by nested-PCR***			
					No. Examined	***L. turanica (%)***	***L. major (%)***	***Mixed (%)***
R. opimus	4 (26.67)	11 (73.33)	15	9 (60)	11	5 (45.45)	0	5 (45.45)
M. libycus	6 (42.86)	8 (57.14)	14	8 (57.14)	14	1 (7.14)	7 (50)	2 (14.29)
Total	10 (34.49)	19 (65.51)	29	17 (58.62)	25		20 (80)	

Discussion

In the current study different epidemiological determinants which facilitates establishment of ZCL in a strategic residential area located at North-East of Isfahan County, were studied. *Ph. papatasi* was the main and dominant species in indoor and rodents borrows of the study area. In our previous entomological study similarly, the number of sand flies captured form rodent's burrows were much more than indoor places and also *Ph. papatasi* was the predominant species either in indoor or outdoor places. (17). *Ph. papatasi* appeared in late May

and disappeared in the late Oct and it appeared in the late Jun and disappeared in the early Oct in outdoors and indoors respectively. *Ph. papatasi* started to appear in the last days of Apr and disappeared in the last days of Oct (17). Same to the results of other studies , the number of captured sand flies in outdoor resting places were more than indoors (18–20), but surprisingly in the current study over 99% of sand flies were captured from outdoors.

Here, natural *Leishmania* infection was detected either by direct observation or nested- PCR in *Ph. papatasi*. *Ph. papatasi* could be infected either by *L. major* or *L. turanica* and mixed infection of both. A previous study showed 37.8% and 18% natural *Leishmania* infection in *Ph. papatasi* and *Se. sintoni* consequently (17). Our previous study showed *Leishmania* infection in *Ph. papatasi* (4.5%), *Ph. caucasicus* (2.3%) and *S. sintoni* (2.8%) (19). During the last two decades several studies confirmed the presence of *L. major* in *Ph. papatasi* by Nested-PCR, Semi Nested-PCR, and Rapd-PCR or isoenzyme (17,21,22).

As the results shows, the majority of captured sand flies in the studied area found un-fed and parous. Other studies conducted previously inside the country showed the majority of dissected sand flies were parous as well (17,19,20).

Phlebotomus papatasi was completely susceptible to deltametrin 0.05%. In agreement to our results, this species found susceptible to pyrthroids including permethrin, deltamethrin, cyfluthrin and lambda-cyhalothrin (7). Additionally, a study conducted in India showed *Ph. papatasi* was resistant to DDT and susceptible to dieldrin, malathion, fenitrothion and propoxur (23). It is hypothesized that, extensive residual spring against malaria vectors in last two decades, has had drastic effect on increasing either tolerance or resistance to DDT and other organochlorines among leishmaniais vectors.

The majority of captured rodents were infected by *Leishmania* using direct examination and nested-PCR consequently. *Rh. opimus* and *M. libycus* play as the first and secondary reservoir host of ZCL in central part of Iran, respectively (5). Natural *Leishmania* infection of *R. opimus* and *M. libycus* by direct microscopic examination, iso-enzyme and molecular technique has been shown by several studies in different parts of the country (16, 24). A study showed the highest (92.9%) and lowest (20%) infection rates of *R. opimus* by *Leishmania* were observed in fall and summer respectively (16). Moreover, it showed *L. major*, *L. gerbilli*, and *L. turanica* circulate in the gerbil population in central Iran, and in agreement with current study *L. major* infection is usually accompanied by *L. turanica* in naturally infected gerbils (16). One study also showed 17.9% natural *Leishmnia* infection among *M. libycus* in central Iran (5).

Incidence and prevalence of ZCL among 965 visited inhabitants in the studied area estimated as 46.55 and 341.97 per 1000 population respectively. In addition, in the same year, scar and ulcer rates calculated as 26.52% and 1.77% among 1184 school children consequently. In a similar study in Qom Province, rates of active lesions in inhabitant were 2.7% and 1.4%, and among school children were 2.79% and 2.26% in 2000 and 2001, respectively (24). In another study in Ardestan County, Isfahan Province, Iran, the rates of scars and ulcers were 3.26% and 1.3% among inhabitant and 0.92% and 1.53% among school children, respectively (18).

Conclusion

Considering high population of gerbils' colonies which offer appropriate microclimate for sand flies breeding, and environmental intervention caused by human and also human dwelling expansion into the natural rodent inhabitant, have led to establishment of the disease cycle among human population in this high risk area. *L. major* is the causative agent, *R. opimus* and *M. libycus* are the reservoir hosts and *Ph. papatasi* is the main vector of the disease in this area. Rodent control and also personal protection such as using impregnated nets and curtains are the appropriate tools in the hands of health authorities to control ZCL in this area.

Acknowledgements

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Conflict of interest

The authors declare that there is no conflict of interest.

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