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Original Article

Seroprevalence of Visceral Leishmaniasis in Children Up To 12 Years Old of Rural Areas from Kermanshah Province, Western Part of Iran

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Abstract

Background*:* After the earthquake in 2017 a few new cases of visceral leishmaniasis (VL) were reported from SarPol-e-Zahab district of Kermanshah Province, western part of Iran. This study was conducted to determine the seroprevalence in Kermanshah Province.

Methods*:* This descriptive cross-sectional study was conducted on children up to 12 years of age from SarPol-e-Zahab County, Kermanshah Province, western part of Iran in 2021. For each individual, a questionnaire including age, sex, clinical features, history of the disease, and contact with canines as reservoir hosts of VL were completed, separately. To determine VL seroprevalence, blood samples were collected from the children and after centrifugation, the sera samples were separated and tested using Direct Agglutination Test (DAT) for detection of anti-*L. infantum* antibodies. Statistical analyses were performed using SPSS16.

Results: Totally, 13 persons were seropositive; 7 samples with titer 1:800, 3 samples had 1:1600, 2 samples had 1:3200 and 1 sample had 1:6400. None of the seropositive cases had a history of kala-azar. There was no significant difference between males and females at titers of anti-*Leishmania* specific antibodies.

Conclusion*:* *L. infantum* infection is being circulated with low prevalence in children up to 12 years old from SarPol-e-Zahab County but it is necessary that the surveillance system is regularly monitored among physicians and public health managers in the studied areas.

Introduction

Visceral leishmaniasis (VL) due to *Leishmani donovani* and *L. infantum/L. chagasi* is a vector-borne neglected tropical disease that is transmitted by female sandflies of *Phlebotomus* (Old World) and *Lutzomyia* (New World) genera (1, 2). This zoonotic infection involves both humans and canine hosts. The areas of endemicity for VL are South America, East Africa, South Asia and the Mediterranean basin; however, over 90% of all human cases occur in six countries, comprising Brazil, India, South Sudan, Sudan and Ethiopia (3, 4). The annual incidence and mortality rate of VL are estimated as 200,000-400,000 and 20,000-40,000, respectively (4). The spectrum of VL clinical symptoms varies from fever and weight loss to anemia, hepatomegaly, splenomegaly and lymphadenopathy (1, 5). Moreover, VL cases in pregnancy have been documented for about a century, reporting harsh sequelae such as miscarriage, abortion and maternal death (6). If left untreated, VL cases may succumb to death, hence it must be considered as a serious tropical disease (7).

Screening of VL cases throughout endemic areas and their timely treatment could be facilitated by early diagnosis using a highly-reliable and reproducible diagnostic method (8). In this sense, the parasitological methods such as microscopy of splenic or bone marrow aspirations are considered as the gold standard, though these approaches are highly invasive despite showing good specificity and require experienced laboratory personnel (9). Molecular polymerase chain reaction (PCR)-based techniques showed promising results in detection of VL agents in human and canine specimens. Reportedly, the Remarkable /High sensitivity and specificity of PCR for identification of *L. infantum/L. chagasi* in human blood samples was calculated to be 93.1% and 95.6%, respectively (10). Nevertheless, there are limitations in using PCR-based assays including lack of standardization due to various molecular protocols as well as expensive equipment rendering them unsuitable for remote areas or field-based studies (11).

A number of serological tests have, also, been developed for serodiagnosis of VL, encompassing latex agglutination test (LAT), the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody test (IFAT), immunoblotting, rK39 rapid test and direct agglutination test (DAT) (12), among which the two latter don't require expensive, sophisticated equipment; hence, they are eligible for screening symptomatic VL cases and field studies (11). Previously, two meta-analysis studies demonstrated high accuracy for DAT in diagnosing VL cases, reporting a sensitivity and specificity of 94.8% and 86% (for studies published during 1986-2004) (13) as well as 96% and 95% (for studies published between December 2004 until April 2019) (11).

The widespread Middle Eastern country, Iran, possesses a subtropical climate which represents favorable conditions for sandfly breeding. The first documented case of human VL in Iran was reported in Mazandaran province in 1949 (14). Since then, some studies have been conducted to detect VL using microscopic and/or molecular methods, particularly from 1998 onwards (15). All over the Middle Eastern territory, *L. infantum* is known as the primary agent of VL which significantly affects children and dogs are the principal reservoir hosts (16). The highest number of VL cases in Iran have been reported from Ardabil and Fars Provinces located in Northwest and South of the country, respectively (1). A recently-published meta-analysis showed that the seropositivity of VL in the general population of Iran is only 2% (95% confidence interval: 1% - 2%) with highest and lowest seroprevalence in northern [3% (95% CI: 1% - 5%)] and western [0.5% (95% CI: 0.2% - 0.7%)] provinces, respectively (14,17). In the

only seroprevalence study in Kermanshah Province, reserchers examined 1800 individuals (1622 samples from >15 children and 178 from adults) using DAT, among which only 6 individuals (0.33%) were seropositive (18). After the earthquake in 2017 in SarPol-e-Zahab a few new cases of VL were reported from this district of Kermanshah province located in western part of Iran.

We aimed to determine the seroprevalence of VL in children under 12 years in rural areas of SarPol-e-Zahab district using DAT during 2021.

Materials and Methods

Ethical considerations:

Informed written consent was obtained from the parents of the children examined. This study was approved by the Research Ethical Review Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1400.082), Tehran, Iran.

Study area

SarPol-e-Zahab city is the third top crowded city in Kermanshah Province (85,342 population), one of the western provinces in Iran, which borders Iraq from its western areas (Fig. 1). In total, the city includes 6 counties and 163 villages. On average, this city is located 550 meters above sea level, having moderate winter and warm summer seasons. The present study was carried out from Oct 2020 to Feb 2021 in 26 selected villages of SarPol-e-Zahab.

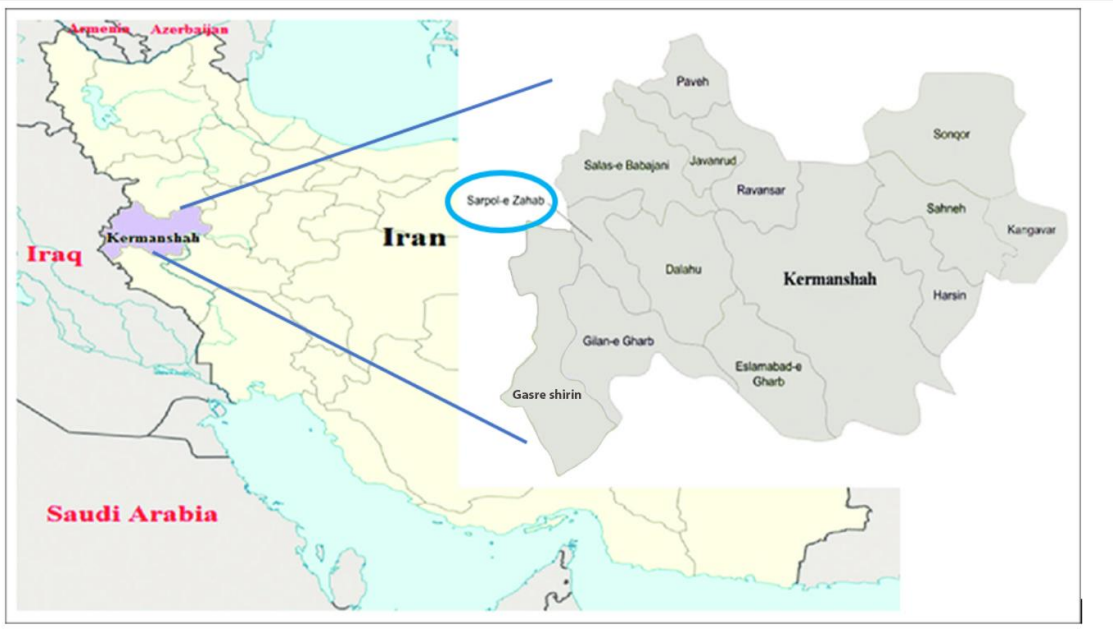

Fig. 1: Situation of Kermanshsh Province in Iran and location of SarPol-e-Zahab in Kermanshsh Province, Iran

Participants and sampling

The study participants were including >12 children of SarPol-e-Zahab City and socio-demographic data such as family name, father's name, age, gender, place of residence, clinical symptoms (fever, anemia, hepatosplenomegaly) and travel history was obtained for each person. Sampling was done upon obtaining the consent of children's parents.

The sample size was calculated as 896, based

on a 95% confidence interval, d = 3% and prevalence (p) = 0.3%.

In order to detect the dispersion of villages, first of all, all the villages were numbered, and then they were separated into two east and west half based on their location on the map. Twenty six villages were randomly selected based on the number and population of villages in two divided parts. Initially, about 2-2.5 ml blood was collected from each examined individual, sera were separated using centrifugation, kept at -20 °C to be further transferred on ice to the Leishmaniasis Laboratory of the School of Public Health, Tehran University of Medical Sciences.

DAT assay

Preparation of DAT antigen

Several steps are performed to acquire DAT antigen, including parasite propagation in RPMI 1640 medium containing 10% fetal bovine serum (FBS), trypsinization of flagellated *L. infantum*, parasite fixation using 2% formaldehyde and staining by coomassie blue 0.02%. After fixation of stained promastigotes in sodium citrate containing 1.2% formaldehyde and by adjusting 50 million promastigotes/ml, the antigen was maintained in dark bottles at 4 °C (19, 20).

Preparation of diluting agents

Briefly, to prepare antigen diluting agent, 0.9 g NaCl and 1 g sodium citrate were dissolved in 100 ml double distilled water. Then, 1.2 ml of the solution was discarded and again 1.2 ml of commercial formalin solution (37%) was added.

In order to prepare human sera diluting agent, 0.9 g NaCl and 0.2 g gelatin powder were dissolved in 100 ml double distilled water, and incubated in a water bath for 30-60 min to be melted. Subsequently, 0.78 ml of the solution was discarded and again 0.78 ml 2-Mercaptoethanol (2ME) was added to eliminate anti-*Leishmania* IgM.

Examination of human sera

First, sera were evaluated at 1/800 dilution for screening and, in case of positive reaction, titration was done to 1/102400 dilution. The experimental plates had 96 (8×12) V-shaped wells. For screening procedure, 8-well rows were considered for one sample, while in case of titration 12-well rows were regarded for one specimen. To prepare sera dilutions, 90 µl human sera diluting agent and 10 µl sera were added to the first well to obtain 1/10 dilution. Subsequently, 10 µl of this mixture was added to the second well and incorporated with 90 µl human sera diluting agent, to obtain 1/100 dilution. In other wells, 50 µl human sera diluting agent and 50 µl human sera were mixed together, in order to yield 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600, 1/51200 and 1/102400 dilutions, respectively. After the addition of 50 µl DAT antigen to the specific well with 1/800 dilution in screening, the plate was incubated at ambient temperature for 13-18 hours. Of note, positive and negative control sera were considered for each set of experiments.

DAT test output

In order to properly read the results, 96-well plates were placed on a white surface. Those wells containing antigen, parasites being observed as a blue dot with distinct margins were considered as negative result (no agglutination), whereas wells with parasites as blue colloid shape were regarded as positive reaction (agglutination). The highest dilution of sample in which agglutination takes place is considered as the highest positive titer. The cut-off titer for dog and human samples is 1/320 and 1/3200, respectively (21). Titration was done for those samples having agglutination during screening process.

Statistical analyses

Chi-square test was used to compare seroprevalence values relative to age, sex, villages, and contact with the dog. Analyses were conducted using SPSS software version 16 (Chi-

cago, IL, USA) with a probability (*P*) value of <0.05 as statistically significant.

Results

Totally, 468 (52%) of 900 of the studied population were male, and 432 (48%) were female. Generally, 13 children were seropositive; 7 samples with titer 1:800, 3 samples had 1:1600, 2 samples had 1:3200 and 1 sample showed 1:6400. All children with antibody titers were asymptomatic. Among the seropositive samples, 7 cases (1.37%) belonged to the 5-8 years old age group (Table 1). There was no significant difference between males and female at titers of anti-*Leishmania* specific antibodies. Although, the number of human cases with titers of antibody in females were higher than males (Table 2). There was significant difference between villages and titers of anti-*Leishmania* specific antibodies (*P*<0.05) (Table 3).

Six months after the first sampling, in order to follow up, children with positive titers were re-sampled, in all of which antibody titers were reduced (Table 3).

Table 1: Seroprevalence *L. infantum* infection by age group in SarPol-e-Zahab district, West of Iran (2020)

Age (yr)	***No. of sample***	***Antibody titer***								***Total of sero-prevalence***	
		1:800		1:1600		1:3200		1:6400		No. of	
		No. of sample	%	No. of sample	%	2No. of sample	0.9%	No. of sample	%	sample	%
≤4	222	1	0.5	1	0.5	0	0	0	0	7	2.36
5-8	296	4	1.4	2	0.7	1	0.3	0	0		
9-12	382	2	0.5	0	0	1	0,3	1	0.3	4	1.04
Total	900	7	0.8	3	0.3	2	0.2	1	0.1	13	1.44

Table 2: Seroprevalence L. infantum infection by gender in children up to 12 years old in SarPol-e-Zahab district, West of Iran (2020)

Sex	***No. of samples***	***Seropositivity***	***%***	***95% Confidence interval (%)***
Male	468	5	1.06	.68-2.7
Female	432	8	1.85	.5-1.2
Total	900	13	1.44	.56-5.38

Table 3: Anti-*L. infantum* antibody titers of thirteen seropositive cases of visceral *L. infantum* infection by direct agglutination test with respect to their age, gender, locality and fallow up children of SarPol-e-Zahab district, 2020

Case No.	***Age (yr)***	***Gender***	***Village***	***Antibody Titer***	***Antibody Titer after follow up***
1	2	Female	Sarab zahab	1:800	1:400
2	3	Male	Gheitek	1:1600	1:400
3	5	Male	Setapan	1:1600	1:400
4	9	Male	Ramaki Ramazan	1:3200	1:800
5	9	Male	Koseha	1:1600	1:800
6	12	Female	Sarab ghale shahin	1:6400	1:800
7	11	Female	Dare balut	1:800	1:400
8	9	Male	Emamieh olia	1:800	1:400
9	7	Female	Emamieh olia	1:800	1:400
10	6	Female	Emamieh olia	1:800	1:400
11	6	Female	Emamieh olia	1:800	No Titer
12	5	Female	Emamieh olia	1:3200	1:800
13	8	Female	Gara Belagh Aazam	1:800	1:400

Discussion

VL is the most malevolent form of leishmaniasis that is considered as a deadly parasitic infection in endemic parts of the world (22, 23). More than 90% of VL cases are it happens in Bangladesh, India, Brazil, Ethiopia, South Sudan, and Sudan which is estimated that about 310 million people are in risk of infection in the counties and 20000 deaths occur annually (24).

VL cases have been reported from most parts of Iran sporadically, however some areas like Ardebil (25), Fars (26), Golestan (27), Bushehr (28), Kerman (29), Qom (30) and North Khorasan (31) have been known as endemic areas to the disease.

As there was no published information about the prevalence of VL in SarPol-e-Zahab, the present study was performed to clarify the status of VL in human. In the present study, direct agglutination test was used, which is known as a valid, inexpensive method, appropriate in field studies and with high sensitivity and specificity (32). Since approximately 99% of seropositive cases in endemic areas are seen on the children less than 12 years old, we performed sampling in this age group.

Results of this study showed that, in 2020, approximately 1.44% of the human samples had antibody titers against *Leishmania* infection. Including 7 samples with titer 1:800, 3 samples had 1:1600, 2 samples had 1:3200 and 1 sample had 1:6400. After the earthquake, people's houses in all the villages of SarPol-e-Zahab were destroyed and people lived in tents, which put them in close contact with dogs as a reservoir and sandflies as carriers of the disease, which could be one of the reasons for the positive cases.

In addition, of the children with antibody titers, only two were under 5 years old, which could be due to less contact with mosquitoes due to the type of cover.

The most positive cases were reported from the village of Emamieh Olia, which had more dogs than other villages and poor sanitation situation. In this study, 13 children had antibody titers, 3 of which had a titer above 1: 3200 and were positive. Of these, 5 were male and 8 were female, but no significant relationship was observed between sex and infection rate. Our results seem to be consistent with the studies of Khorasan, Lorestan and Bushehr based on the relationship between sex and the rate of infection (31, 33, 34). Also, contrary to the results of our study, in some studies, including Ardabil and Qom, a significant difference was observed between the sex and the rate of infection (35, 36). It is necessary to mention that the diagnosis of symptomatic disease, asymptomatic disease and past infection is not possible using DAT (20).

Our results showed that there was a significant association between positive serum rates and villages in SarPol-e-Zahab City, which is consistent with a study conducted in Alborz province (37). In our study, people with antibody titers had not previously had Kala-azar.

The presence of visceral leishmaniasis in the province requires health facilities to prevent, diagnose and treat this disease. It is also necessary to increase awareness and vigilance among the people, doctors and public health managers, especially in high-risk rural areas of the province.

Conclusion

L. infantum infection is being circulated with low prevalence in children up to 12 year ages from SarPol-e-Zahab County but it is necessary that the surveillance system is regularly monitored among physicians and public health managers in the studied areas.

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Conflict of interest

The authors declare that there is no conflict of interest.

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