

Original Article

Human visceral leishmaniasis: Seroprevalence survey of asymptomatic adults in an endemic area of Northwestern Iran

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ABSTRACT

Background & Aim: Visceral leishmaniasis (VL) or kala-azar is a protozoan disease caused by some species of *Leishmania donovani* complex. Mediterranean type of the disease is endemic in various parts of Iran. A cross-sectional study was designed to determine the seroprevalence of VL among asymptomatic adult population in Meshkin-Shahr area from the Northwest of Iran as an endemic focus of VL.

Methods & Materials: Altogether, 180 blood samples were collected from asymptomatic adults' population throughout 2015. Before sampling, a questionnaire was separately completed for each individual. All the collected blood samples were examined by direct agglutination test (DAT) after plasma separation. Anti-*Leishmania infantum* antibodies at titers 1:100 to 1:1600 was considered as *L. infantum* infection, while the cut-off titer of $\geq 1:3200$ with specific signs and symptoms was considered as VL.

Results: From 180 collected plasma samples, nine (5%) of them showed anti-*Leishmania* antibodies at titers 1:400 and higher. Distribution of anti-*Leishmania* antibodies titers was 1:400 (n = 2), 1:800 (n = 4), and 1:1600 (n = 3). All of the seropositive cases were observed among females. All the seropositive individuals had not a history of kala-azar. The highest seropositivity rate was observed among the age group of 13-23 years old. No changes in titers of anti-*Leishmania* antibodies observed after collected the seropositive blood samples again and tested by DAT with 1-month interval.

Conclusion: Visceral *Leishmania* infection is relatively high among adult people reside in Meshkin-Shahr area without any clinical manifestations. Asymptomatic VL infection is very important in immunocompromized individuals such as HIV-positive cases; these patients are at risk to manifesting clinical signs and symptoms of VL. Therefore continuing serological surveillance for detection of visceral *Leishmania* infection should be recommended in the endemic foci of VL.

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Introduction

Visceral leishmaniasis (VL) is a protozoan disease can be produced by *Leishmania donovani* complex such as *Leishmania infantum* in the Middle East and Mediterranean area (1). The disease generally transmitted by the bite of the female sand fly. Canines are main reservoir hosts of VL in Mediterranean region (1). VL is the most severe form of leishmaniasis in the world. The annual occurrence of human VL worldwide is estimated 500,000 and about 59,000 deaths (2) and approximately 100-300 in Iran (3). Since the last decade, the incidence rate of Mediterranean type of VL has increased, mostly because of *Leishmania*-HIV coinfection (4). In humans, *L. infantum* causes several clinical signs and symptoms, from asymptomatic or oligosymptomatic infection to acute or chronic disease (5). The clinical signs and symptoms of the disease are as follows: fever, hepatosplenomegaly, substantial weight loss, progressive anemia, and death (6). VL has a high mortality rate in untreated patients, malnourished cases and in cases of VL/HIV coinfection (7). Diagnosis of VL is done by parasitological methods (demonstration of amastigotes in biopsies of aspirates or by culture of parasite), serological tests, and molecular methods. Parasitological method is painful, and molecular methods need advanced lab equipment but serological tests, particularly direct agglutination test (DAT), do not require advanced lab equipment in comparison with other methods. Hence, they are completely

appropriate for use under field conditions (8). In this study, DAT was used to detect of seropositive samples because it is simple, valid, and cost-effective test (sensitivity and specificity of the DAT were calculated 92-100% and 72-100%, respectively) (2, 3, 9). The presence of the first case of VL in Iran was reported from the Mazandaran Province in 1949 (10). Since 1980, annually more than 100 human cases have been diagnosed from the Northwest of Iran, mostly, from Meshkin-Shahr areas among children under 12-year-old (3). It shows that VL has been endemic in this area for a long time (11). This study was conducted to determine the seroprevalence of VL among adults' people in above-studied areas among the adult population. It was carried out from February 2015 to January 2016 for making aware of present status and endemicity of VL in this area and selection and implementation of optimal control program if necessary.

Methods

Meshkin-Shahr District is located in Ardabil Province, Northwest of Iran (Figure 1). Sabalan, one of the highest and famous mountains of Iran is located near the city. The city of Meshkin-Shahr is situated at an altitude of 1490 m above the sea and the weather of it is moderate mountainous. It covers an area of approximately 1530 km² including 323 villages and its population is estimated to be 169,967 among which 42% was settled in urban areas and 58% in rural areas (12).

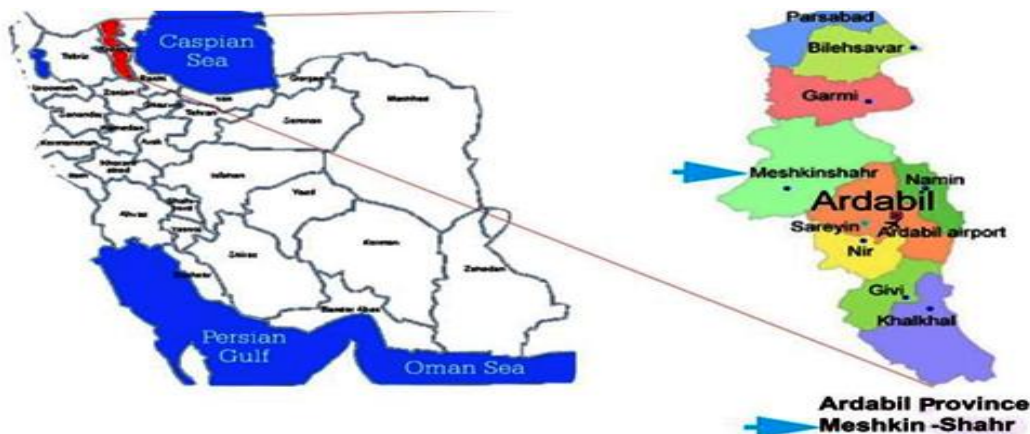


Figure 1. Geographical situation of Meshkin-Shahr District in Ardabil Province, Northwest of Iran

This descriptive cross-sectional study was conducted during February 2015 to January 2016 for 1 year. A simple randomized sampling method was used for the sample collection. A questionnaire was completed by each subject, recording sociodemographic characteristics (age groups, gender, location, occupation, history of VL, clinical symptoms, and directly contact with domestic dogs). Blood samples were taken from 180 adults (≥ 13 -year-old) subjects randomly. Subjects were consisting of 33 (18.3%) male and 147 (81.7%) female, samples were collected in ethylenediaminetetraacetic acid-coated tubes and processed them 4-10 hours after collection, then samples centrifuged at $2000 \times g$ for 5 minutes and then plasma was collected in individual micro tubes in order to DAT examination and were stored at $-20^{\circ} C$ until tested. The *L. infantum* antigens for this study were made in the Parasitology Unit of the School of Public Health in the Tehran University of Medical Sciences. The first step of preparing DAT antigen was the production of promastigotes mass of Iranian strain of *L. infantum* [MCAN/IR/07/Moheb-gh. (GenBank Accession No. FJ555210)] in RPMI1640 medium (Biosera, South America) in addition 10% fetal bovine serum (Biosera, South America), following trypsinization of the parasites, staining with Coomassie brilliant blue R-250 (Sigma, USA) and fixing with formaldehyde 1.2% (9, 13, 14). All plasma samples were tested by DAT. The procedure described by Mohebali et al. followed for titration of *Leishmania*-specific antibodies (9). Overall, for screening of samples, three dilutions of 1:800, 1:1600 and 1:3200 were made and tested. The samples that were positive with titer 1:400 and higher were diluted up to 1:102400 in a V-shaped microtiter plate. Plasma diluent (physiological saline) contains 0.78% b-mercaptoethanol and 0.2% gelatin. Then, 50 μ l of antigen suspension was added to each well. After 1 minute of gentle shaking on a flat surface, the plate was covered and stayed near wet cotton as a wet room in temperature of 21-25 $^{\circ}C$, and then results were read after 18-24 hours. The highest dilution, which is agglutination, was still recognizable in comparison with positive and negative controls

titer was defined as the titer of the sample. Compact blue dots with specified margin were defined as negative subjects, and large diffuse blue mass was defined as positive subjects. Antigen control well (antigen and diluent plasma only) and negative and positive controls were used for comparison in each plate daily. Titers of $\geq 1:3200$ were considered as seropositive (9, 15-19) the cutoff was based on the previous studies (15, 18, 20). The finding was analyzed by SPSS software (version 19, SPSS Inc., Chicago, IL, USA), with a $P < 0.05$ considered as statistically significant. Chi-squared and Fisher exact tests were followed to comparison of prevalence values relative to the characteristics of subjects.

Results

Of the 180 plasma samples collected from adults' subjects of Meshkin-Shahr district, 9 cases (5%) showed anti-*Leishmania* antibodies in at titers 1:400, 1:800, 1:1600. Two cases presented a titer of 1:400, four had a titer of 1:800, and three cases had a titer of 1:1600 (Table 1).

Table 1. Results of DAT s on 180 plasma samples collected from adult population from Meshkin-Shahr District, in 2015

Number of examined	Antibody titer			Total
	1:400	1:800	1:1600	
180	No 2 (1.1%)	No 4 (2.2%)	No 3 (1.7%)	No 9 (5%)

DAT: Direct agglutination tests

The frequency of anti-*Leishmania* antibody titers with DAT according to the age groups can be seen in figure 2. In this study, anti-*Leishmania* antibodies were not detected in titer $\geq 1:3200$, any clinical symptoms observed in all seropositive subjects, and nobody had the previous history of the disease or remembered it in the past.

The study populations were consisting of 33 (18.3%) male and 147 (81.7%) female, with age ranging from 13 to 79 years. Most of the cases were aged 23-33 years. Age, gender and the rate of antibody titers distribution of seropositive subjects can be seen in table 2.

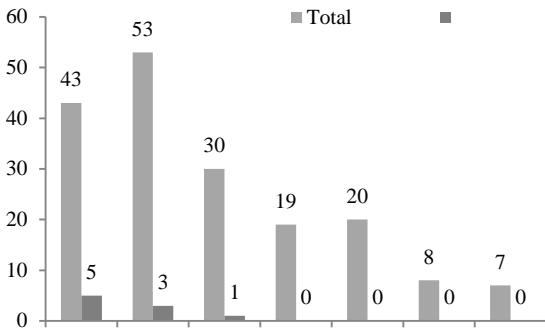


Figure 2. Results of direct agglutination tests from 180 collected plasma samples by age group

All of the seropositive cases were observed in females group. The highest proportion of positive subjects was observed in 13-23 years group. There was no significant difference between the rate of seropositivity and other sociodemographic characteristics (age groups, gender, location, occupation, history of VL, clinical symptoms, and contact with animals) ($P > 0.05$). The occupation of 8 seropositive subjects was household and one was a student.

In the present study after a month, five out of nine seropositive subjects were tested again and followed up for anti-*L. infantum* antibodies, but none differences observed between in recent and previous tests. Due to any apparent clinical symptoms has been observed in seropositive subjects, we resulted that subjects just contact with the parasite but they do not get any active disease, therefore, the present investigation found asymptomatic subjects in the study region.

Discussion

This study is the first seroepidemiological

survey of *Leishmania* infection among adults in one of VL-endemic area in the Northwest of Iran, Meshkin-Shahr District. This study aimed to assess the rate *Leishmania* infection in adults; therefore, 180 samples have been prepared from Meshkin-Shahr area, in Ardabil province.

Findings of this study revealed that 5% of healthy cases in Meshkin-Shahr area are infected by *L. infantum*. In spite of the persistence of visceral infection, more than 90% of *L. infantum* and *L. donovani* infection do not progress to clinically apparent diseases (21), but HIV-positive or immunosuppressed status cases might be at the risk of acquiring clinical disease. Due to the observation of any clinical symptoms in seropositive subjects, this study found asymptomatic cases. Based on seroepidemiological surveys which were carried out in Iran, asymptomatic forms of VL caused by *L. infantum* were more than symptomatic ones (15), and considerable number of infected individuals only manifested a mild, oligosymptomatic disease that resolves without treatment (22).

In this study, DAT was used for detection of *L. infantum* infection, according to previous study, the result of DAT is valid and reliable and showed with highly in specificity and sensitivity (23).

The results of DAT showed in spite of 5% of cases in the studied areas had anti-*Leishmania* antibodies in titers of 1:400 ($n = 2$), 1:800 ($n = 4$), 1:1600 ($n = 3$), none of them were DAT positive at titers 1:3200 and higher as cutoff values of DAT on human sera. The sensitivity and specificity of this method (DAT) vary in different studies between 90-100% and 72-100%, respectively (9).

Table 2. Results of DATs nine seropositive cases by age, gender and location

Case No.	Age (years)	Gender	Living place from Meshkin-Shahr area	Antibody titer	Sign and symptom
1	42	Female	South	1:1600	No
2	23	Female	Center	1:1600	No
3	29	Female	East	1:1600	No
4	14	Female	Center	1:800	No
5	18	Female	West	1:800	No
6	32	Female	Northwest	1:800	No
7	27	Female	Center	1:800	No
8	23	Female	Northwest	1:400	No
9	21	Female	Center	1:400	No

DAT: Direct agglutination test

A serological study was conducted by Mohebbali et al. (23). During 2002-2005 in some VL-endemic parts of the Iran, that results were as follows: In Ardabil Province, Meshkin-Shahr (3.6%), Germe (2.8%), Ardabil, Pars-Abad and Khalkhal (5.1%), in Chahar Mahal and Bakhtiari Province, Koohrang (2.3%), in Fars Province, Mamasani (1.9%), in Lorestan Province, Poshtkuh (1.3%), in Kohgiluyeh and Bouir ahmad Province, Yasuj (1.5%), in Khorasan Province, Bojnurd and Shirvan (0.46%). In Qom Province, Fakhar et al. (24) found 1.7% of seropositivity in 8 villages of Ghahan that three of seven seropositive cases had the previous history of VL and majority of the cases were among children among 7-10 years of age. In this study, all of the seropositive cases had not any previous history of VL. Mohebbali et al. (25) found 3.4% sero-positivite subjects in Dashti and Dashtestan districts of Bushehr Province. Another serological survey was made by Mahmoudvand et al. (10) in Baft district, Kerman Province, in this study from 1476 subjects, in only 23 cases (1.55%) observed anti-*Leishmania* antibodies at titers of 1:800 and 1:1600 and just in 14 cases (0.95%) observed anti-*L. infantum* antibodies at titers of $\geq 1:3200$. According to the mentioned study, VL is endemic in the Northwest of Iran especially in Meshkin-Shahr District and according to the results of study *L. infantum* infection is relatively high in Meshkin-Shahr District. Furthermore, in our study, there was no significant difference between the rate of seropositivity and other sociodemographic characteristics (age groups, gender, location, occupation, history of VL, clinical symptoms, and contact with animals) ($P > 0.05$). In some of the previous studies conducted in Iran, there has been no relatively in consistency of the data between males and females (23, 24). Although any clinical disease by *L. infantum* has been reported in adults in Iran so far, there are so many reports in other endemic countries such as case reported in Turkey. A total of 14 cases (7 female, 7 male) diagnosed as VL in Adana and Anatolia, Turkey, and followed for 1 year. 10 of the cases were immunocompetent and four were immunosuppressive. Clinical symptoms in

these cases have been observed as followed: fever (57%), splenomegaly (79%) and hepatosplenomegaly (50%). The diagnosis of VL was made by the observation of amastigote in the smears of bone marrow aspiration (26). In another study in Turkey, data of 12 VL-patients in Hassan II, Fez Hospital was collected during to January 2009 to January 2014 (27). A 31-year-old male patient, who was the first adult VL case from Zonguldak (a province located at western Black-Sea region of Turkey), was reported in another survey. He was hospitalized for 2 months with a history of fever, chills, sweating, and weight loss (28). In Iran, there are a few studies about adults suffering from *L. infantum* infection.

Between 2004 and 2006, 802 apparently healthy subjects, in the Northwest and SouthEast of Fars province in Iran, were tested for anti-*Leishmania* antibodies, by DAT, and for *L. infantum* kinetoplast DNA, in polymerase chain reaction (PCR)-based assays. Generally of the 426 cases from Northwestern Fars, by DAT, 8 cases were found positive and by PCR 68. Of the 376 cases from South-Eastern Fars, by DAT 5 cases were found positive and by PCR 32 (29). A total of 2003 healthy blood donors in five VL-endemic districts in Fars province, southern Iran, were collected and tested for antibodies against *L. infantum* by DAT. 28 blood donors (1.4%) were positive for *Leishmania* infection by DAT (30).

After a month, the 9 seropositive cases were tested again but had not developed in clinical and none differences observed in the recent and previous test.

Conclusion

Our findings indicated that visceral *L. infantum* infection is still relative prevalent in Meshkin-Shahr area in spite of control programs that have been implemented during last decades. Due to the importance of VL in immunocompromized patients, the surveillance system with DAT should be continued in the endemic foci of VL in Iran.

Conflict of Interests

Authors have no conflict of interests.

Acknowledgments

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