Original Article

A Sero-Epidemiological Study on Visceral Leishmaniasis among Volunteer Children and Adults in Rural Areas of Shahroud, Iran 2018–2019

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Abstract

Background: Visceral leishmaniasis (VL) also known as Kala-azar is considered as one of the zoonotic infections in Mediterranean countries. The disease reservoir and vectors are dogs and sandflies respectively. Due to reported sporadic cases of Kala-azar in the past five years in Shahroud County, Semnan Province, Iran, this study aimed to investigate the status of this infection in this area and to determine its seroepidemiology to take required measurements for infection control and treatment.

Methods: This study was conducted on 504 subjects residing in seven villages in Shahroud County. Blood samples were randomly selected using the cluster sampling method and were collected from subjects aged up to 13 years old (90%) and adults over 13 years old (10%) from September to May 2019. After separating sera from whole blood, samples were subjected to direct agglutination test (DAT) to detect anti-*Leishmania infantum* antibodies. A range of 1:10 to 1:800 dilutions were prepared from the samples.

Results: Results of 1:800 titration indicated that no sample was positive for antibodies against *L. infantum*. After the secondary screening, 10 cases (1.98%) showed the antibody titer of 1:100, while four cases (0.79%) showed the antibody titer of 1:400. Of 14 cases with the *L. infantum* antibodies, all were detected from the children <13 years old. According to clinical findings, no patient was suffering from fever, weight loss, splenomegaly, hepatomegaly, and cachexia and therefore did not show the Kala-azar symptoms.

Conclusion: The results of the current study indicate that Kala-azar is not prevalent in Shahroud County.

Keywords: Visceral leishmaniasis; Seroepidemiology; Direct agglutination test; Human; Iran

Introduction

Protozoan parasitic infections are considered major health problems globally. Leishmaniasis is one of the most common contagious infections in tropical regions, and it is considered as one of the leading causes of death due to parasitic infections after malaria (1-2). This infection is observed in different cutaneous, mucocutaneous, and visceral forms. Mucocutaneous forms are not common in Iran; however, visceral leishmaniasis (VL) also known as Kala-azar is observed sporadically or endemically. Kala-azar is a systemic infection, mainly transmitted to humans by sand flies from the genus *Phlebotomus* in the Old World (3-5). The incidence of this infection has been reported at 500,000 cases and the fatality subsequent to this disease has been estimated at 75,000 globally; however, VL is mostly found in developing countries with poor hygiene (6).

Symptoms in humans include prolonged

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fever, hepatosplenomegaly, weight loss, pancytopenia, fatigue, and delayed treatment may result in death due to secondary infections or internal bleeding. In most cases, Kala-azar may not directly lead to death; nonetheless, patients with the suppressed immune system and HIV patients may contract this infection leading to complications and death (4, 7-8). Kala-Azar is mostly observed in young children below the age of 12 years old, and it is most common in rural areas. Acute forms of infection are mostly present in children aged < 2 years old or immunocompromised patients and the chronic forms are mostly observed in healthy adults. The gold standard for diagnosis of VL is the observation of amastigotes in aspirates of spleen or bone marrow. However, in endemic areas, serological testing is more common (9-10).

The reservoir of Mediterranean VL, is majorly dogs or beavers, and the etiological agent for this type of infection is mostly *Leishmania infantum* which is present in Iran although there is no evidence for its etiology (11-14). In Iran, the first case of VL was reported by Pouya, in 1949 in rural areas of Tonekabon, in the western region of Mazandaran Province, North of Iran. Ever since, VL has been endemically reported in some regions in Iran such as Fars Province, Eastern Azarbaijan, Ardabil and Boushehr (15). The presence of *Ph. neglectus*, *Ph. keshishiani*, *Ph. kandelakii*, *Ph. alexandri*, *Ph. major*, and *Ph. tobbi*, known vectors of *L. infantum*, in Iran highlights the global distribution and significance of these species in the transmission of leishmaniasis (16-21). To our knowledge, no epidemiological studies have been conducted on the prevalence of this infection in Shahroud County, Semnan. Epidemiological studies are highly important as they would provide insight into the status of the infections and thereby, help us take the necessary measures for infection control and treatment. Therefore, this study aimed at investigating the epidemiological status of VL and determining the seroprevalence of this infection in Shahroud County located in Semnan Province.

Materials and Methods

Study area

This cross-sectional descriptive study was carried out from September 2018 to May 2019 to determine the seroepidemiology of VL in both children with < 13 years of age and in adults residing in Shahroud County. Shahroud County in Semnan Province is located in the east of Tehran, at the junction of the road between Mashhad and Gorgan. Regarding the climate of the city, it has a dry desert climate with cold winters and hot summers. Rain is not frequently seen as the city ends in the central desert of Iran on the southern side. The average precipitation is 167.7mm a year and its average relative humidity is 48% during a year. This county, with a population of 218628 is the most populated county in Semnan Province.

Study design

In this study, a randomized cluster sampling was carried out corresponding to seven villages, namely Dehmolla, Abarsij, Moghan, Abr, Bedasht, Haq ol Khvajeh, and Kalateh Motahhar from all geographical regions of the County (Fig. 1). The sample size of 504 patients was estimated based on Cochran's method. As only 454 children the age of <13 years old volunteered for the test, 50 volunteered persons the age of >13 years old with suspected clinical manifestations were randomly chosen for the sampling. Subsequently, multistage sampling was carried out and informed consent was taken from every subject and demographic information of each subject, including age, sex, clinical symptoms, and any disease background was recorded. All subjects were permanent residents of the mentioned regions.

For blood collection, 50µl of blood samples were collected from subjects at Rural Health

Care Centers by disposable sterile lancets and capillary tubes that were non-heparinized. Blood samples were then transferred to the Department of Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, in cold chain conditions to prevent the disruption of antibodies and hemolysis. Sera were then separated using centrifugation at 3000rpm for 5min. All sera were then stored at -20 °C for further tests. For serological tests, sera were tested by direct agglutination test (DAT) to detect antibodies against *L. infantum* according to the methods previously described by Harith et al. (22). In this serological study, antigens used in DAT are the promastigote forms of *L. infantum* (23). If the antibodies against *L. infantum* are present in the sera of infected patients, the antigens and antibodies form a mixed blueish suspension without sedimentation. On the other hand, in case antibodies against *L. infantum* are absent, a dark blue spot is observed at the bottom of the plate following the accumulation of DAT antigen. For the DAT test, serial dilutions of sera were prepared with 0.9% saline solution, 0.2g gelatin (1.04070/ Merck, Germany), 0.78ml 2-ME (M-6250/ Sigma, USA), and 100ml sterile distilled water at 1:10–1:800 in V-Bottom polystyrene microplates. Then, 5µl of DAT antigen was added to each well. *Leishmania infantum* antibodies were used as positive control and the sera of a *L. infantum*-free person was used as a negative control to avoid any possible test errors including autoagglutination and so on. Finally, after 12–18 h incubation at room temperature, the plates were assessed for antibody agglutination, and the test was repeated one more time for any suspicious agglutination.

For initial screening, dilution of 1:800 of samples was used for antibody detection. According to previous studies, antibody titer of ≥ 1:3200 in samples of patients with clinical manifestations is indicative of Kala-azar, while antibody titer of 1:800 and 1:1600 are considered negative and suspected of infection, respectively. In cases of samples suspected of VL, sampling should be carried out again 2–3 weeks later to confirm the results. In case a significant increase is observed in antibody titer, VL is confirmed. In case no positive result was observed in the initial screening using 1:800 titration, all sera were screened again using lower titers. Finally, results from DAT and demographic data were analyzed using SPSS software v. 22. Fisher's exact and Chi-square tests, with a 95% confidence interval, were performed to compare values relative to age, gender, and region. The existence of statistical significance was assumed if $P \leq 0.05$.

Results

This study was conducted on a total of 504 subjects residing in seven villages in Shahroud County. Of 504 subjects, 302 (60%) cases were male, and 202 (40%) cases were female. The age range of the participants was 3 months to 59 years old. In fact, 90% of subjects were up to 13 years old and 10% of subjects were 13 or above 13 years old. Serum samples were screened at dilution of 1:800. At the initial screening stage, using DAT, no antibody titer of ±1:800 was found, however, after the secondary screening, 10 cases (1.98 %) showed an antibody titer of 1:100, while four cases (0.79%) showed the antibody titer of 1:400. Demographic data are presented in Table 1.

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Table 1. Demographic characteristics of the participants and the geographical distribution of subjects showing 1:100 and 1:400 titration with DAT antibodies against *L. infantum* in 2018–2019 in Shahroud

Characteristics	No.	Percent	1:100 titration (No.)	1:400 titration (No.)
Gender				
Male	302	60		
Female	202	40		
Age group				
≥13 years old	50	10		
2-13 years old	411	82		
<2 years old	43	8		
Resident area				
Dehmolla	75	14.8	1	
Abarsij	75	14.8	2	1
Moghan	100	19.8	2	1
Abr	100	19.8	3	2
Bedasht	75	14.8	2	
Haq ol Khvajeh	40	7.9		
Kalateh Motahhar	39	7.7		

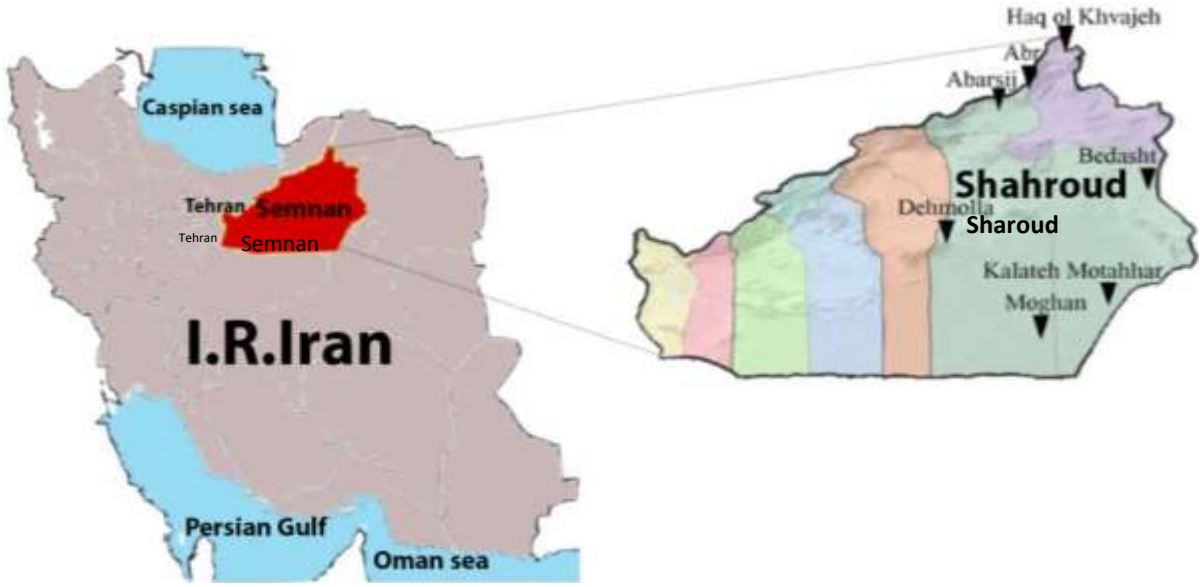

Fig. 1. Schematic map of Shahroud and the geographical locations of sampling villages

Discussion

Visceral leishmaniasis (VL) also known as Kala-azar is a deadly parasitic disease if left untreated, especially in infants, and a global public health concern. This infection has been reported sporadically or endemically in some regions in Iran (15). Kala-azar has several types of which Mediterranean Kala-azar has been reported in Iran. Since there were unreported sporadic incidences of infection with *L. infantum*, we aimed at investigating the status of VL in this region. However, the following two studies have shown there was no VL sand fly vector species (*Ph. Kendelaki*, *Ph. tobbi*, *Ph. major*, *Ph. perfiliewi*, and *Ph. alexandri*) in the region (Shahrood and Damghan where is very close to Sharood) (24, 25). The clinical mani-

festations of Kala-azar like fever, weight loss, splenomegaly, hepatomegaly, and cachexia were absent in the individuals, and the subclinical infection might be present in asymptomatic patients and DAT can be used for the detection of antibodies against *L. infantum*. Generally, for epidemiological studies, DAT is commonly employed in endemic areas in large-scale screening of VL. This technique is simple, valid, and cost-effective with high sensitivity and specificity of 90–100% and 72–100% respectively (26, 27).

We could not find any study regarding the prevalence of VL in Shahroud County; however, there have been several other studies that have investigated the prevalence of VL using serological testing in other regions in Iran. During a four-year period of 2002–2005, a survey of 6558 subjects from different regions of Iran known as endemic regions indicated the prevalence of 4.9 and 4.4% of VL in northeastern and northwestern Iran, respectively. The prevalence of this infection was lower in the central and southwestern parts of the country with rates of 1.5% and 1.9%, respectively (15).

Our study showed zero prevalence of VL in Shahroud which is somehow in accordance with the rates reported in different parts of Iran. In another study by Mahami et al. (2003) 1155 children up to 12 years old and adults in Ardabil were tested for VL using DAT. According to their results, 32 cases (2.8%) showed the antibody titer of 1:800, while 7 cases (0.6%) were serologically positive for VL with the antibody titer of 1:3200 (28).

In 2013, the clinical implications, as well as the epidemiology of VL, were studied in different parts of Iran using DAT. A total of 1698 cases showed the anti-*L. infantum* titer of 1:3200 and these cases were mostly aged up to 12 years old and almost 75% of cases with antibody titer of 1:3200 were in endemic areas and were having clinical manifestations (15).

In 2011, 402 human blood samples were taken from Mazandaran Province and tested for VL using DAT. Among these subjects, 8 cases (2%) showed the antibody titer of 1:1600 and were therefore suspected as VL cases. The results showed no definite positive case of VL which is in accordance to the current study (29). On the other hand, in 2015, blood sampling was conducted on 800 subjects residing in Lorestan Province, and the prevalence of VL was determined using DAT. The anti-*L. infantum* titer was 1:800 and 1:1600 in 21 cases (2.62%) and 5 cases (0.62%) showed the antibody titer of 1:3200 of which only one case had the clinical manifestation of anemia (30).

In several Kala-azar endemic regions in Iran and the globe, most cases of VL infections are in the asymptomatic form. In another study in Iraq, 20% of children who were either healthy or asymptomatic showed anti-*Leishmania* antibodies by the DAT method. The existence of infected patients who are showing no symptoms of Kala-azar in VL endemic regions may affect the transmission cycle of the parasite responsible for this infection. Therefore, evaluating the residents in these regions by employing serological testing such as DAT can facilitate the on-time identification of VL patients to reduce the rate of transmission (31). Similarly, in 2015, in a survey, 1007 blood samples of children under 10 years old residing in rural areas of Alborz Province were collected and 37 children showed anti-*Leishmania infantum* antibodies with titers of ≥ 1:800 and 2 children showed anti-*Leishmania infantum* antibodies with titers of ≥ 1:3200 with clinical features and then treated with anti-leishmaniasis drugs in pediatric hospital (32). On the other hand, in 2017, blood samples were collected from 617 children up to 12 years old residing in rural areas of Fars Province with no clinical manifestations and 17 cases showed antibodies against *L. infantum*, and the highest rate of infection was observed among children aged 5–8 years old, indicating the subclinical VL in children from rural areas in Southern Iran (33).

According to the results of the current study and the presence of sub-clinical cases, it seems that the prevalence of VL in Shahroud County

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is very low. Increased availability of healthcare centers and improved policies of the country's health system to control diseases transmitted by insects play a major role in increasing or decreasing the incidence of this infection. According to multiple studies in endemic areas of VL in Iran, not only symptomatic dogs, but also asymptomatic dogs can harbor *L. infantum* infection, thereby affecting the transmission cycle in endemic areas. Therefore, it seems necessary to conduct additional epidemiological studies on reservoirs to prevent the spread of the disease in these regions.

Conclusions

The results of the current study indicate that Kala-azar is not circulating in Shahroud County. Additional epidemiological studies are required on vectors and reservoirs of *L. infantum* to prevent any sporadic incidences in the region.

Acknowledgments

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Ethical considerations

To maintain ethics, before sampling, informed consent was obtained from the parents of all children participating in the study. This study had the approval code IR.TUMS.SPH.REC.1397.129 from the Research Ethical Review Committee of Tehran University of Medical Sciences, Tehran, Iran.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this paper. This research was conducted in an objective and unbiased manner, and the authors have no financial or personal relationships that may have influenced the results or interpretation of the data presented.

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