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Original Article

Seroepidemiological Study of Visceral Leishmaniasis (Kala-azar) in Children under 12 Years Old in North of Iran: An Observational Study in 2019-2020

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Abstract

Background: Visceral leishmaniasis (VL) caused by the *Leishmania donovani* complex that is transmitted by the bites of female sandflies. Mediterranean type of VL caused by *L. infantum*. While, Roudbar County of Guilan Province has been introduced as a suspected cutaneous leishmaniasis focus; there are no published data on the seroprevalence of VL in Guilan Province. We aimed to investigate the seroprevalence of this disease in Roudbar County.

Methods: This descriptive cross-sectional study was carried out in 2019-2020 among children less than 12 years of age to determine the seroprevalence of VL by direct agglutination test (DAT). Blood samples were randomly collected from 918 children under 12 years of age refers to the public health center in the clusters.

Results: Out of 918 children, 14 (1.52%) showed anti-*Leishmania* antibodies, with 4 (0.43%), 2 (0.21%), 8 (0.87%) anti-*L. infantum* antibodies at titers 1:800, 1:1600 and ≥1: 3200 respectively. All children with anti-*Leishmania* antibody titers of ≥1:800 were evaluated by a physician. Clinical manifestation of VL including fever, anemia and hepatosplenomegaly observed in a 6-year-old boy from Defraz village with anti-*Leishmania* antibody of titers ≥102400.This patient was admitted to the pediatric hospital in Rasht, capital of Guilan province, Iran and was successfully treated.

Conclusion: VL is being circulated with low prevalence in children up to 12 years old in Roudbar, northern part of Iran. Accordingly, it is critical to improve the awareness of physicians and public health supervisors about the importance of this fatal disease in Guilan province and especially in Roudbar area.

Introduction

Visceral leishmaniasis (VL) is endemic in more than 60 countries in tropical and subtropical areas, and about 200-300 thousand new cases occur every year. In Iran, VL of the Mediterranean type is endemic and the principal agent of this disease is *Leishmania infantum* (1, 2). Infection with this parasite has also been reported in dogs, jackals, foxes and wolves (1, 3). VL is endemic in some parts of Iran including Fars, Ardabil in the northwest, East Azerbaijan, Bushehr, North Khorasan, Chaharmahal & Bakhtiari and Lorestan provinces; also, it has been reported sporadically in other provinces of the country. VL is detected mostly in infants with the general signs of fever, hepatosplenomegaly, paleness, lymphadenopathy and blood cell reduction. These signs usually followed by inside hemorrhage or secondary infection and lead the person to his death (4-7).

The gold standard of diagnosis of VL remains the detection of *Leishmania* amastigote form of the parasite in tissue samples such as spleen, liver, lymph nodes and bone marrow, which are sensitive but invasive and potentially hazardous. It seems that serological methods can be a good alternative to laboratory diagnosis of VL. Direct agglutination test is a valid, scientific, cost effective and highly specific and sensitive method for laboratory diagnosis and epidemiological studies of VL (8, 9).

While Roudbar County of Guilan Province has been introduced as a new leishmaniasis focus (10), there are no published data on the prevalence of VL in Guilan Province.

Considering that children are generally influenced by this infection, we conducted a seroepidemiological investigation of VL in children less than 12 years of age in Guilan province using DAT.

Materials and Methods

Geography of the study area

Guilan area in the Caspian Sea littoral of northern Iran is surrounded by Mazandaran Province in the east, Ardebil Province in the west and Zanjan and Qazvin provinces in the south. It is also bordered by Azerbaijan Republic in the north as well as Russia across Caspian Sea. Guilan area with calm climate and moderately warm-humid summer is located between 36o 33'–38o 27' N latitude and 48o 32'–50o 36' E longitude and formally composes 16 counties. Most areas of the province with about 1000–2000 mm annual rainfall have the highest precipitation in Iran and rice is the main agricultural crop. Roudbar County located in Guilan Province with about 200–500 mm annual rainfall and showed mountainous and less humid temperate climate similar to the Mediterranean region. The county has an area of 2574km2 and the main agricultural crop is olive (10). At the 2006 census, the county's population was 101,884 in 27,902 families. The county has seven cities: Rudbar, Barehsar, Jirandeh, Lowshan, Manjil, Rostamabad and Tutkabon (11) (Fig. 1).

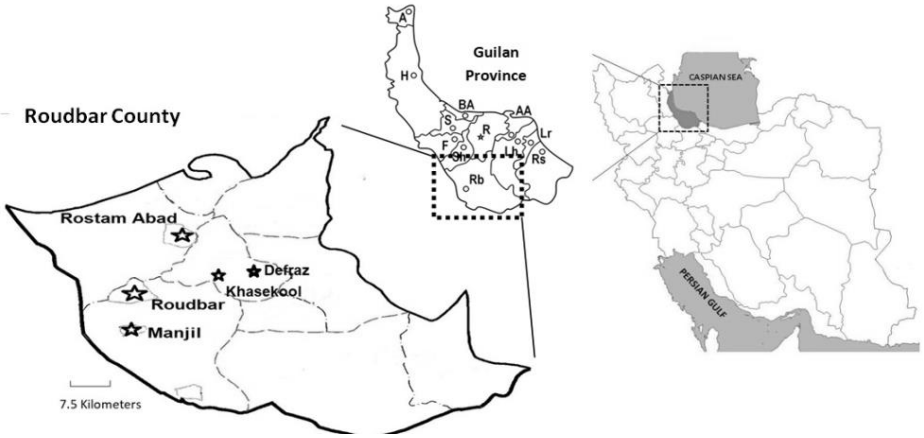

Fig.1: Map of Iran highlighting Guilan Province- North of Iran, surveyed in 2019-2020

Subjects and Blood Collection

This descriptive and cross-sectional study was done in 2019-2020. All areas of the reported VL cases in Roudbar County (including 2 villages and 3 cities) were applied for sample collection. Cluster random sampling was applied for sample selection. All health care centers in the area that were suspect of VL were selected. Regarding two villages (Khaskool and Defraz) all children less than 12 years were included. For 3 cities, all children less than 12 years refer to public health centers in the study area included.

Ethical approval was obtained through the Guilan University of Medical Sciences with an ethics number of IR.GUMS.REC.1398.286 for the sampling and use of the blood samples and questionnaire information.

Approximately 200 µl of blood samples were collected from each child and were centrifuged at 200 g for 5 min, and the collected sera were stored at -20°C in department of parasitology, Guilan University of Medical Sciences. Finally, all the serum samples were analyzed using DAT in the Leishmaniasis Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. In addition, for each of the subjects, questionnaires including personal characteristics such as age, sex, location, clinical signs, travel history and contact with dogs were recorded.

Direct agglutination antigen (DAT)

The DAT antigen was obtained via multistage procedure, according previous study, which included mass production of promastigotes of the Iranian strain of *L. infantum* [MCAN/IR/07/Moheb-gh (GenBank accession no. FJ555210)] in RPMI-1640 medium (Biosera, South America) plus 10% fetal calf serum (Biosera, South America), following trypsinization of the parasites, staining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, Missouri, USA) and fixing with 1.2% formaldehyde (12). Finally, all collected plasma samples were tested by DAT according to the previous study in Iran (12). The cut-off titer was defined as ≥1:800 for *Leishmania* infection, in accordance with the previous study. Samples that were positive as 1:800 were diluted on a V-shaped microtiter plate up to 1:102400 in a dilution liquid containing %0.9 saline and %0.78 mercaptoethanol. An equal volume (50 µl) of antigen suspension was added to each well. The results were read after 18-24 hours of incubation in a humid room at room temperature. The highest dilution at which clumping is observed is regarded as the HA titer of the sample. Compact blue dots were considered as negative water mattresses and large and colloidal dots as positive.

Titer of ≥1:3200 along with specific symptoms are considered as confirm disease. Titers 1:800 to 1:1600 are also considered as *L. infantum* infection.

The seropositive children were referred to a pediatric infectious disease specialist for further clinical examinations and treatment.

Statistical Analysis

SPSS software ver.16 (SPSS Inc, Chicago, IL, USA) was used for data analysis. The chi-square and Fisher's exact tests with confidence level of 95% were used to compare seroprevalence values relative to demographic parameters.

Results

In total, 918 blood samples from children up to 12 years old (51.4% male and 48.6% female) were collected from urban and rural areas of Roudbar county. Out of 918 children, 14 (1.52%) showed anti-*Leishmania* antibodies, with 4 (0.43 %), 2 (0.21%), 8 (0.87%) anti-*L. infantum* antibodies at titers 1:800, 1:1600 and ≥1: 3200 respectively (Table 1, 2).

Regarding different study areas, the infection rate was 1.5%, 0.6%, 2.38%, 2.31% and 2.5% in Roudbar, Rostamabad, Manjil, Defraz and Khaskool respectively.

The discoveries of leishmaniasis seroprevalence in subjects in the 2 rural and 3 cities of Roudbar County are indicated in Table 2.

Table 1: Seroprevalence of human visceral leishmaniasis by direct agglutination test in the 2 rural and 3 urban districts of Guilan Province, north of Iran

Antibody titer	***Locality***					***Total***
	Manjil city	Rostamabad city	Roudbar city	Khaskool village	Defraz village	
1:800	2	0	2	0	0	4
1:1600	1	0	0	0	1	2
≥1:3200	1	1	4	1	1	8
Total	4	1	6	1	2	14

Table 2: The seroprevalence of *Leishmania* infection in the 2 rural and 3 urban districts of Guilan Province, north of Iran

Sampling sites	***No. of samples***	***Seroposivity***				***Total***	***P value***
		1:800 No. (%)	1:1600 No. (%)	1:3200 No. (%)	1:5200 No. (%)		
Roudbar city	422	2 (0.47)	0 (0)	2 (0.47)	2 (0.47)	6 (1.42)	*P*>0.005
Rostamabad city	190	0 (0)	0 (0)	1 (0.52)	0 (0)	1 (0.52)	
Manjil city	176	2 (1.13)	1 (0.56)	1 (0.56)	0 (0)	4 (2.27)	
Defraz village	89	0 (0)	1 (1.12)	0 (0)	1 (1.12)	2 (2.24)	
Khaskool village	41	0 (0)	0 (0)	0 (0)	1 (2.43)	1 (2.43)	
Total	918	4 (0.43)	2 (0.21)	4 (0.43)	4 (0.43)	14 (1.52)	

In the present study, all of the people with anti-*Leishmania* antibody titers of ≥1:800 were surveyed by a physician, and a 6-year-old boy from Defraz village with anti-*Leishmania* antibody of titers ≥1:102400 indicated clinical manifestation of VL (with symptoms of fever, anemia, hepatosplenomegaly) was admitted to the pediatric hospital and was successfully treated.

In addition, the highest and the lowest rates of infection were observed within the 5-8 and 9-12 age groups respectively (Table 3).

In terms of gender, the incidence of the infection was the same in both males and females and was not statistically significant (Table 4). In any case, no association between sex and titer of antibodies against *Leishmania* was founded. Using Chi-square test, no significant difference was observed between the seroprevalence of the parasite in the sampled areas with a history of travel (*P*>0.005).

Table 3: The seroprevalence of *Leishmania* infection in different age groups in the 2 rural and 3 urban districts of Guilan Province, north of Iran

Age	***No. of Samples***	***Seropositivity***				***Total***	***P value***
		1:800 No. (%)	1:1600 No.(%)	1:3200 No. (%)	1:5200 No. (%)	No. (%)	
4	141	1 (0.70)	0 (0)	1 (0.70)	1 (0.70)	3 (2.12)	*P*>0.005
5-8	295	3 (1.01)	2 (0.67)	2 (0.67)	1 (0.33)	8 (2.71)	
9-12	482	0 (0)	0 (0)	1 (0.2)	2 (0.41)	3 (0.62)	
Total	918	4 (0.43)	2 (0.21)	4 (0.43)	4 (0.43)	14 (1.52)	

Table 4: The seroprevalence of *Leishmania* infection in males and females in the 2 rural and 3 urban districts of Guilan Province – North of Iran

Sex	***No. of Samples***	***Seropositivity***				***P value***
		1:800 No. (%)	1:1600 No. (%)	1:3200 No. (%)	1:5200 No. (%)	
Male	471	2 (0.42)	1 (0.21)	3 (0.63)	1 (0.21)	P>0.005
Female	447	2 (0.44)	1 (0.22)	1 (0.22)	3 (0.67)	
Total	918	4 (0.43)	2 (0.21)	8 (0.87)	14 (1.52)	

Discussion

Visceral leishmaniasis is endemic in some parts of Iran. Since the status of this disease was not known in Guilan Province, this study was performed to explore the prevalence of VL in Roudbar county. This is the first study which preliminary investigates the seroprevalence of VL in Guilan province. Approximately 1.52% of the randomly chosen children had antibody titers of ≥1:800 against *Leishmania* infection. The rate of *Leishmania* infection at titers ≥1:800 noted in the present study was lower than those reported in Bojnord of Khorasan Province (2.4%), Germi of Ardebile Province (2.8%), Baft of Kerman Province (2.5%) and Alborz Province (3.7%) (12-15).

Meanwhile, some studies reported that the rates of VL among subjects with an antibody titer of ≥1:3200 were 0.56%, 0.6%, 0.33% and 0.4%, in some areas of Iran including Bojnord, Germi, Kermanshah and Alborz respectively (12, 13, 15, 16) which are lower than findings of our study (0.87%).

In the present study, the larger parts of the seropositive cases displayed no clinical symptoms. They were considered asymptomatic cases. VL deliver a wide extend of clinical appearances from asymptomatic to deadly cases in Iran (4, 12, 16), and elsewhere in the world, as well as, in India, Turkey, Croatia, and Brazil (17, 18).

Longitudinal follow-up studies have indicated that some cases eventually develop clinical VL, but most will never develop the disease and does not require treatment (12). In the present study, all of the individuals with anti-*Leishmania* antibody titers of ≥1:800 were evaluated by a physician, and in a 6-year-old boy from Defraz village with anti-*Leishmania* antibody of titers ≥1:102400 clinical manifestation of VL including fever, anemia, hepatosplenomegaly was registered. This child was hospitalized and treated successfully. These clinical features were similar to findings of other studies conducted within the Mediterranean region (12, 19-21). In our study, despite the occurrence of few signs and symptoms such as fever, anemia, and weight loss in other children with anti-*Leishmania* antibodies, during referee to physician and follow-up examination, none of them showed any progressive VL clinical signs.

The highest and the lowest rates of infection were observed within the 5-8 and 9-12 age groups respectively.

Similar to the present study, VL was more common in 5-8 years age group in Baft of Kerman province (14).

Antibodies against leishmaniasis, which are detected by a DAT test, stay in the blood a few years after cure. Therefore, a decrease in the antibody titer of these people who are infected at a younger age will be seen at the age of 9 years and older (22).

Furthermore, no positive results were observed in serum in children under 1 year because they are probably less exposed to mosquito, type of their coverage and the long incubation period of VL (23).

In terms of gender, the incidence of the infection was the same in both males and females and was not statistically significant. This finding was in accordance with those detailed in other parts of Iran (16, 24). In another study, the relationship between sex and infection with *Leishmania* parasite was not reported to be significant (16). In a study in the Brazilian city of Sierra (25) and India, both females and males were equally exposed to infection before puberty and after puberty due to the existence of female sex hormones this ratio is lower in women (26).

Using Chi-square test, no critical contrast was observed between the seroprevalence of the parasite in the sampled areas with a history of travel.

Among suspicious cases of the present study, only one person had travel history and the others had not left the area for at least 6 months before our study began.

In general, most of the suspected and infected people did not travel to leishmaniasis endemic and non-endemic areas. Therefore, due to the presence of the reservoirs (dogs and canines) and vectors (sandflies) of VL in the region (10), it could be concluded that they have probably been infected in Roudbar county.

In a previous study on the *Phlebotomine* fauna in Roudbar County, the existence of vectors of leishmaniasis was proven. Noteworthy, all collected *Phlebotomus* species were suspected or proven vectors of CL and/ or VL in Iran or adjoining countries (10).

Results indicated seropositive individuals in all study areas. Presence of livestock, domestic dogs, wild canids and VL vectors provide an appropriate condition for disease transmission.

The use of PCR for detection of *Leishmania* DNA in wild sand flies is a useful technique for the identification of putative *Leishmania* vectors in different geographical zones. The most preferences of molecular strategies are their sensitivity and specificity, independent of the number, stage and location of the parasite in the insect midgut (27). The study of vectors

and reservoirs by molecular specific tests for *Leishmania* parasite in Guilan Province is recommended.

Since this study performed on children under 12 years old and in areas where the cases of VL had already been reported, the results could not be extended to all population living in Roudbar County.

However, due to the importance of this fatal disease, it is necessary to improve the awareness of physicians and public health supervisors regarding avoidance, determination, and treatment of this disease. Furthermore, it is critical to improve the awareness of physicians and public health supervisors about the importance of this fatal disease in Guilan province and especially in Roudbar area.

Conclusion

This is the first study which confirms the existence of Kala-azar in Guilan province and proves the circulation of visceral laeishmaniasis among children under 12 years old in Roudbar County.

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Conflict of interest

The authors declare that there is no conflict of interest.

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