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Original Article

Seroprevalence of Canine Leishmaniasis in Sheltered Dogs in Bushehr Province, Southwest of Iran during 2022-2023

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Abstract

Background: The Mediterranean form of visceral leishmaniasis (VL) is endemic in some regions of Iran and is often seen in children under 10 years old. There is a 90% mortality in patients, if diagnosis and treatment are not done on time. Canids, as reservoirs, play an important role in the spread of the disease.

Methods: Bushehr Province, southern Iran is always mentioned as one of the endemic areas for VL, so for this purpose, as the first study in the region, 112 sheltered dogs in Bushehr City were evaluated for canine leishmaniasis (CanL) using serological Direct Agglutination Test (DAT) as well as detailed clinical examinations.

Results: Out of 112 samples collected, 71.4% of cases had anti-*L. infantum* IgG antibody titers of 1:80 and higher and therefore considered as seropositive. Furthermore, from the 70 seropositive dogs with antibody titer of 1:320 and higher, 47 (42%) had at least one of the clinical symptoms associated with VL and considered as cases with CanL.

Conclusion: The current seroprevalence situation of dogs in this region, is very noticeable and can be an important alarm for policymakers and health system practitioners. More comprehensive and complementary parasitological studies should be carried out on a number of reservoirs in the region for diagnosis and treatment and to accurately determine the statistics of the disease compared to the obtained seroprevalence status.

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Introduction

Visceral leishmaniasis (VL) or kala-azar is seen sporadically in most areas of Iran and is endemic in some regions of Ardabil, East Azerbaijan, Fars, Qom and Bushehr provinces. Different species of *Leishmania* such as *L. infantum*, *L. donovani* and *L. chagasi* have the ability to cause the visceral form of the disease. In Mediterranean region, including Iran, the main causative agent is *L. infantum* and the reservoirs are dogs and canines (1). According to the WHO report, an estimated currently 50,000 to 90,000 new cases occur worldwide annually, which are thought to be underestimated. VL in Iran is often seen in children under 10 years of age and has a wide range of symptoms (asymptomatic to acute and fatal cases). The main and important symptoms of the disease are long-term fevers, splenomegaly and anemia. Also, pancytopenia, hyper-gammaglobulinemia, and hypo-albuminemia are unusual findings in the blood samples of the patients. There is a 90% mortality in patients, if diagnosis and treatment are not done on time (2-4).

In the Mediterranean form of VL, which is the only form of the disease in endemic areas of Iran, stray dogs can act as reservoirs of the disease, and even despite the observance of many health standards, these animals cause the spread of VL in these regions. Bushehr province is always mentioned as one of the endemic areas for VL, but so far no study has been done regarding the potential reservoirs of this disease in the region. Bushehr province also has the largest common land border with Fars province, which is considered as one of the most important known centers of the disease in the country; so during the years 1996-2002, more than 520 disease cases were reported from all over Fars province (5).

Different methods can be used to diagnose VL in reservoirs. The diagnosis based on the clinical symptoms observed in the animal does not have sufficient sensitivity and is not efficient, because the degree of adaptation of the parasite to dogs is much higher than to humans, and in many infected dogs, there may be no specific clinical symptoms for years, and gradually the disease becomes progressive and manifests as cachexia, skin swelling and itching, hair loss, returned paws, hepatosplenomegaly, lymphadenopathy, inactivity, anemia and finally, the appearance of skin lesions and wounds on the edges of the eyelids and muzzle of the animal (6-8). Parasitological methods are very difficult and time-consuming and are based on direct sampling from the animal and microscopic detection of the parasite, which often lead to the death of the animal, and in addition, sometimes even though the reservoir is infected, it is not possible to find parasites in the samples taken. However, as a suitable alternative, different serological techniques can be used for this purpose. The direct Agglutination Test (DAT) has high sensitivity and specificity and due to its simplicity, accuracy and reliability, it has been introduced as one of the selective tests in diagnosis and widely used during the last three decades for the serological diagnosis of the visceral form of leishmaniasis. Based on the result of a research, this method is recommended for serodiagnosis of visceral leishmaniasis in human, especially in endemic areas (9,10).

In Bushehr, one of the endemic regions of the country, there is no report on the status of dogs being infected with *L. infantum* and for this purpose, as the first study in the region, stray dogs sheltered in dog shelters in Bushehr city were evaluated for CanL and their potential in spreading this very important disease.

Materials and Methods

Ethical approval

This study was approved by the Ethical Committee of Bushehr University of Medical Sciences with Ethics Code: IR.BPUMS.REC.1401.141.

Study Area

Bushehr Province is located in southwestern Iran. The province's population exceeds one million people, of which 71.5% live in cities and 28.5% in villages. The geographical coordinates of this region is between 27° and 19' to 30° and 16' north latitude and 50° and 1' to 52° and 59' longitude, and the mean annual temperature is 25.7 °C. The weather in the province is warm for 7 months during the year, moderate-cold for 2 months and mild-warm for 3 months. The population of the city is close to three hundred thousand people and this area has hot and humid climate most of the year (Fig. 1).

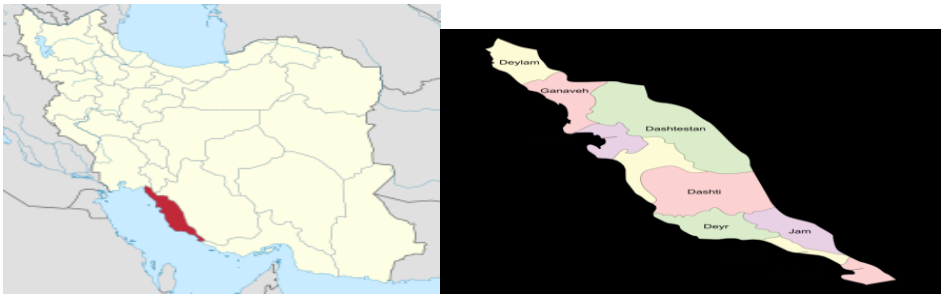

Fig. 1: a. Geographical location of Bushehr province (red), b. Map of Bushehr province by cities

Sample Collection

The studied population was the number of 112 dogs kept in dog shelters in Bushehr city, regardless of gender, age, and breed, which were selected totally between March 2022 and March 2023. After coordination with the municipal officials, by repeatedly referring to the dog keeping camps at the mentioned time intervals, clinical examinations of each of the studied dogs were done by a qualified veterinarian and their characteristics were recorded in data forms. Then, about 5 ml of venous blood was taken from each dog. The collected samples were sent to the Iran Leishmaniasis Reference Laboratory located in the Department of Parasitology, Faculty of Health, Tehran University of Medical Sciences, keeping the cold chain.

Preparation of parasite antigen

Briefly, after mass culture of *L. infantum* in RPMI-1640 culture medium and washing three times in PBS buffer, the precipitate was mixed with trypsin and incubated at 37 °C for 45 minutes. After washing again, it was mixed with the same volume of 2% formalin and set at a fixed number ($200x10^6$) and kept for 20 hours at 4 °C. After the end of incubation and centrifugation, the sample was washed three times with citrate solution and kept in brilliant Coomassie blue dye for 12 nights at 4 °C. The sample was washed again with citrate solution and finally the number of $50x10^6$ parasites was adjusted in the diluting solution. The container containing the antigen solution was covered with aluminum paper and kept at 4 °C until the test (11).

Direct Agglutination Test (DAT)

DAT test was performed on the studied samples in two stages of screening and then titration for positive samples, in 96-Vshaped well microplates. In summary; after diluting the sera by 10 times with the diluting solution and preparing serial dilutions to 8 dilutions in the wells, the samples were incubated for 1 hour in a humid chamber. Then, 50 microliters of the antigen solution prepared in the previous step was added to each of the wells, and after rotating for 1 minute, was incubated again in a humid chamber for 18 to 24 hours (12).

Interpretation of the DAT Test

After the incubation period, the tests were read and interpreted; in this way, the observation of lump-shaped accumulation in the center of the well is a sign of non-agglutination and, in other words, the test is negative, and the observation of uniform blue colloidal color in the well liquid indicates agglutination of the reaction and a positive test. Based on previous studies, dilutions of higher than and lower than 1:80 were classified as seropositive and seronegative samples, respectively. Also, dilutions of 1.320 and higher with clinical symptoms were considered as infected cases (13,14).

Statistical Analysis

The test results and questionnaire data were analyzed using SPSS statistical software (Chicago, IL version 16, SPSS Inc.) and the chi-square test.

Results

Out of 112 samples collected, 80 cases (71.4%) had anti-*L. infantum* IgG antibody titers of 1:80 and higher and therefore considered as seropositive. The remaining 32 samples (28.6%) lacked acceptable antibody titer and were evaluated as seronegative for CanL (Table 1).

Table 1: Frequency distribution of anti-*L. infantum* IgG antibodies in the serum of sheltered dogs in Bushehr camps

Ab Titer	n	%	Result
1:320 and higher	70	62.5	Seropositive
1:80-1:320	10	8.9	Seropositive
Lower than 1:80	32	28.6	Seronegative
Total	112	100	

The examined samples were grouped in 3 age ranges: under one year, 1-3 years and 3-5 years. The most seropositive cases were detected in the age group of 1 to 3 years (53.5%), although the highest evaluated population was also in this age group, and statistical analysis did not show a significant relationship between the status of infection and the age of the studied dogs (Table 2).

Eighty-seven of the examined dogs (77.7%) were male and the rest (22.4%) were female. Statistical analysis showed that there is no significant relationship between the gender of the studied dogs and their seropositivity. Also, the rate of seropositivity has no significant relationship with the size of the investigated dogs (Table 2).

Table 2: Frequency distribution of anti-*L. infantum* IgG antibodies in the serum of sheltered dogs in Bushehr camps based on the age, sex and size

Results	Seropositive		Seronegative		Total		*P.* value
	n	%	n	%	n	%	
Age(yr)							
≤ 1	7	6.3	2	1.8	9	8.1	0.653
1-3	60	53.5	25	22.3	85	75.8	
3-5	13	11.6	5	4.5	18	16.1	
Gender							
Male	59	52.6	28	25	87	77.6	0.253
Female	21	18.8	4	3.6	25	22.4	
Size							
Small	18	16.1	3	2.7	21	18.8	0.153
Big	53	47.3	29	25.9	91	81.3	

Various clinical symptoms related to VL were also evaluated and the possibility of a logical relationship between positive tests and symptoms was analyzed using appropriate statistical tests. In general, from the number of 70 seropositive dogs with 1:320 and higher titer, 47 (42%) had at least one of the clinical symptoms associated with VL, but in dogs with no clinical symptoms, this rate was much lower (23; 20.5%) (Table 3).

Table 3: Frequency distribution of anti-*L. infantum* IgG antibodies in the serum of sheltered dogs in Bushehr camps based on clinical symptoms in general

Results	Clinical Symptoms				Total	
	yes		no			
	n	%	n	%	n	%
Seropositive (1:320 and higher)	47	42	23	20.5	70	62.5
Seropositive (1:80-1:320)	6	5.4	4	3.5	10	8.9
Seronegative (Lower than 1:80)	16	14.3	16	14.3	32	28.6
Total	69	61.7	43	38.3	112	100

As shown in Table 4, no significant relationship was found between the seropositivity of the examined dogs and some variables such as skin wounds and hyperkeratosis lesions, hepato-splenomegaly, lymphadenopathy, cachexia, lameness and lethargy. However, hair loss in seropositive dogs was far more than seronegatives, which is noticeable; although this difference was not significant in the statistical analysis (Table 4).

Table 4: Frequency distribution of anti-*L. infantum* IgG antibodies in the serum of sheltered dogs in Bushehr camps based on some clinical symptoms

Variable	Seropositive		Seronegative		Total		*P.* value
	N	%	N	%	N	%	
Skin wounds							
Yes	40	35.8	14	12.5	54	48.1	0.836
No	40	35.8	18	16.1	58	51.9	
Cachexia							
Yes	7	6.3	2	1.7	9	8	0.502
No	73	65.2	30	26.8	103	92	
Lameness							
Yes	18	16.1	10	8.9	28	25	0.615
No	62	55.3	22	19.7	84	75	
Hair loss							
Yes	50	44.7	14	12.4	64	57.1	0.191
No	30	26.8	18	16.1	48	42.9	
Lymphadenopathy							
Yes	9	8	5	4.5	14	12.5	0.423
No	71	63.4	27	24.1	98	87.5	
Hepato-splenomegaly							
Yes	8	7.1	1	0.9	9	8	0.222
No	72	64.3	31	27.7	103	92	
Lethargy							
Yes	34	30.4	13	11.5	47	41.9	0.479
No	46	41.1	19	17	65	58.1	

Other variables investigated in this study were muzzle sores, nasal septum sores, eye sores, nosebleeds, and extended claws, which in none of the above-mentioned cases, there was no statistically significant relationship.

Discussion

Visceral leishmaniasis is caused by different strains of *Leishmania* parasite and is seen sporadically in most regions of Iran and endemic in some regions including Bushehr province. In Iran, this disease is often seen in children under 10 years of age, and if the treatment is not done on time, there is a 90% chance of death. In the Mediterranean form, which is the only VL form found in the endemic regions of Iran, stray dogs can act as reservoirs of the disease, and even though many health standards are observed in a region, these animals cause the spread of VL in these regions (1,2,15).

Bushehr province has always been mentioned as one of the endemic areas for VL, but until now, no study has been done regarding the infection status of dogs as reservoirs of the disease. For this purpose, in this research as the first study in the region, stray dogs in dog shelters in Bushehr city were evaluated by DAT method.

In general, out of 112 samples collected, 80 cases (71.4%) had anti-*L. infantum* IgG antibody titers of higher than 1:80 and therefore considered as seropositive. The remaining 32 samples (28.6%) lacked acceptable antibody titer and were evaluated as seronegative for CanL. The amount of seropositive cases obtained in this study is very remarkable and compared to similar studies conducted in other regions of Iran, it is very high. Of course, considering the history of VL in this province and referring to reliable sources that Bushehr province is always mentioned as an endemic region for VL, obtaining a high percentage of seropositive dogs of the region was not far from the expectation. But in any case, the prevalence of over 70% in the region is very impressive and can be an important alarm for policymakers and health system practitioners in the region.

In a similar study that was conducted to determine the seroprevalence of VL on 92 stray dogs in Khorramabad, Iran, only 3 dogs (3.26%) had IgG antibodies against *L. infantum*. The researchers have acknowledged that VL has a low prevalence in stray dogs in the Khorramabad, but despite this, it indicates the presence of infection in the population of these dogs and can be the cause of disease transmission to people in Khorramabad area. Based on this, the monitoring of leishmaniasis in dogs and the management of the stray dog population have been considered important in preventing the transmission of leishmaniasis and other diseases that can be transmitted to humans (16).

In a study conducted to investigate visceral leishmaniasis in children and adults from 13 villages and nomadic tribes in Bushehr province during 1998-1999, the number of 1496 plasma samples were evaluated using DAT method and seropositive rate found 3.4%. Authors have suggested the elimination of stray dogs, identification and treatment of infected humans and herd dogs to control of visceral leishmaniasis in this endemic area (15).

In another study conducted in pet dogs of three cities (Kerman, Baft and Bam) of Kerman Province, the overall seroprevalence in a total of 116 dogs was obtained to be nearly 13% (7.4, 18.75 and 23.5% in Kerman, Baft and Bam cities, respectively). The authors have considered the prevalence of infection in Baft City to be predictable according to the previous study conducted in this city and also have mentioned the situation of Bam City as noteworthy. Finally, they have noted the necessity of additional ecological studies on the reservoirs and vectors of the disease in different regions of Kerman Province due to the reports of VL cases in children of Baft, Jiroft and Kahnouj cities (17).

In a serological study in stray dogs of Zahedan City, randomly 150 stray dogs were evaluated using the IFA method after a complete clinical examination. The overall seropositivity of evaluated dogs in this research was estimated to be around 5.3%. This study has shown the low seroprevalence of VL in dogs of Zahedan as compared to other geographical areas of Iran, which indicates the absence of VL in this region. The authors' suggestion has been to conduct further studies on high-risk populations and disease reservoirs and vectors in the region (according to the reports of the presence of *Phlebotomus* species in the province) (18).

In another study to determine the serological prevalence of VL in herd dogs of Kahnouj City in southern Iran, 94 herd dogs were evaluated using a commercial ELISA kit, and according to the researchers, none of the samples were positive in the serological examination. The authors have introduced that Kahnuj herd dogs do not play a role as reservoirs of VL and transmission of the disease to humans in this region (19).

In the study carried out for the purpose of serological investigation of VL in dogs in the southwest and central part of the country, blood was collected from 548 stray and owned dogs in Tehran, Chaharmahal, Bakhtiari and Khuzestan provinces, and the sera have been evaluated by direct agglutination method (DAT). The results indicated the presence of anti-*L. infantum* IgG antibodies in 53 cases (nearly 10%) of the samples, which the highest amount was obtained in Chaharmahal and Bakhtiari province with 16%. Based on the obtained results, authors have recommended the necessity of serological screening for VL in dogs without clinical symptoms as one of the disease control strategies (20).

In the present study, 87 (77.7%) of the examined dogs were male and the rest (22.3%) were female. Statistical analysis did not show a significant relationship between the gender of the researched dogs and their positive serology. Also, no significant relationship was found between the size of the dogs and the positivity rate. These findings are consistent with the findings of some similar studies (17,20).

Another finding of this study was that there was no significant relationship between the positive serology of the examined dogs and some variables such as skin wounds and hyperkeratosis lesions, hepato-splenomegaly, lymphadenopathy, cachexia, lameness and lethargy. Other variables investigated in this study were the muzzle sores, nasal septum sores, eye sores, nosebleeds, and extended claws, which in none of the above-mentioned cases, there was no statistically significant relationship. But the incidence of hair loss in dogs with positive serology was much higher than dogs with negative serology, which is noticeable. In summary, out of 70 seropositive dogs with higher than 1:320 titer, 47 (42%) had at least one clinical symptom related to VL. According to the rules and standards, samples with an antibody titer of 1.320 and above along with clinical symptoms are considered as sick and need treatment and health care. In research conducted in Kerman province, out of 116 dogs investigated, only two cases had hair loss and skin lesions in the muzzle and around the eyes (17).

Relying on the very high prevalence (71.4%) obtained in the present study, it is possible to expect a change in the status of the disease in the region from the current endemic state to being introduced as a hyper endemic region in the new epidemiology classification of this disease in Iran. However, due to the fact that no previous study in this field has been conducted in the region, therefore, it was not possible to compare the results of the present study with the previous statistics, and it is not possible to make a more logical decision about the course of the disease and whether the rate of VL seroprevalence has increased in reservoirs of the region or has had a constant trend in recent years.

Conclusion

The current situation obtained in the region during this research is very important and can be an important alarm for policy makers and health system practitioners in the region. Therefore, emergency and necessary measures should be taken from the planners and health officials of the region to prevent and control the disease before the peak of the life cycle of the parasite between the reservoirs and vectors in the area and the transmission of the disease to the residents.

It is suggested that more comprehensive and complementary parasitological studies be carried out on a number of reservoirs in the region for diagnosis and treatment and to accurately determine the statistics of the disease compared to seroprevalence status, and therapeutic measures as well as solutions to interrupt the life cycle of the parasite and control and prevent of VL should be implemented.

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Conflict of interest

Authors declare that they have no conflict of interest.

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