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Visceral Leishmaniasis in Stray Dogs from Kermanshah Area, Iran: Seroprevalence and Association With Clinical and Hematological Alterations

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Abstract

Background: Visceral leishmaniasis (VL) is responsible for mortality, especially among children in developing countries. Stray dogs are reservoir of VL infection. Infected dogs that are asymptomatic can act as a source of infection for humans.

Objectives: This study aimed to investigate the seroprevalence of visceral leishmaniasis in stray dogs from Kermanshah area and also evaluate clinical and hematological alterations in dogs naturally infected with *Leishmania infantum*.

Methods: Ninety-two stray dogs aged from 1 to 8 years old were sampled. Serum samples were evaluated for the presence of anti-*Leishmania infantum* antibodies using enzyme-linked immunosorbent assay (ELISA). All positive samples were titrated by direct agglutination test (DAT).

Results: In total, 11 (11.95%) of the examined dogs were infected with *L. infantum*. Among seropositive dogs, only 4 cases (36.36%) showed clinical signs. Three infected cases had anemia, while two had hemoconcentration. According to blood count, most alterations were observed with MCHC, band neutrophils, and lymphocytes.

Conclusion: The high frequency of asymptomatic dogs indicates that these reservoirs must be considered as the principal source of VL infection in this area. To decrease disease incidence in

human, frequent surveillance and monitoring of canine visceral leishmaniasis (CVL) is critical, especially in stray dogs.

Keywords: CBC, Clinical signs, ELISA, *Leishmania infantum*, Stray dogs

1. Introduction

Leishmania species are digenetic protozoa that cause a zoonotic disease in some mammalians called leishmaniasis (Silva *et al.*, 2018). The parasite species are transmitted by *Phlebotomus* spp. sandflies. *Leishmania infantum* is the main causative agent of visceral leishmaniasis (VL) in Iran (Moradi-Asl *et al.*, 2020; Najafi *et al.*, 2021). Domestic dogs (*Canis familiaris*), especially stray dogs, are considered the most important reservoir host for *L. infantum* (Estevam *et al.*, 2022; Carneiro *et al.*, 2023; David Ola-Fadunsin *et al.*, 2023).

Canine visceral leishmaniasis (CVL) in most infected dogs is asymptomatic which carry the parasite and can act as a source of infection to other hosts including humans (Silveira *et al.*, 2021; Chiyo *et al.*, 2023). The clinical signs of CVL may appear over a long period of time, from 3 months to 7 years post-infection, and include hepatomegaly, dermatitis, anorexia, cachexia, ocular lesions, onychogryphosis, and cutaneous ulcerations (World Health Organization, 2010; Sousa *et al.*, 2016).

Currently, CVL is common at least in some districts of more than half of the provinces of the country (Razzaghi Manesh *et al.*, 2012). The prevalence rate of CVL in Iran varies from 2.6% to 93.3% from endemic to non-endemic regions (Shokri *et al.*, 2017). Canine visceral leishmaniasis is partly resistant to routine therapeutic protocols that are used for people. Therefore, one of the best measures to prevent human infections is rapid identification and control of infected stray dogs (Chiyo *et al.*, 2023; de Castro *et al.*, 2022). Although laboratory tests abnormalities are usually unspecific in CVL, they are important for diagnosis, staging of the disease, therapeutic monitoring, and determination of the prognosis (Nicolato *et al.*, 2013). Hematologic alterations commonly reported in CVL are mild to moderate normocytic-normochromic anemia, neutrophilia, and mild to moderate thrombocytopenia (Almeida *et al.*, 2021; Paltrinieri *et al.*, 2016).

Detection of anti-*Leishmania* antibodies is the method of choice for mass screening in epidemiological studies (Osuna *et al.*, 2022). Currently, several diagnostic methods including indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), direct agglutination test (DAT), and western blotting have been described for the detection of anti-*Leishmania* antibodies in human and canine sera (Olfaty-Harsini *et al.*, 2017; Duthie *et al.*, 2018; Pessoa-e-Silva *et al.*, 2019; Fujisawa *et al.*, 2021). The present study aimed to investigate the

seroprevalence and risk factors of CVL in stray dogs of Kermanshah using ELISA and DAT, and also to evaluate the hematological alterations in dogs naturally infected with *L. infantum*.

2. Materials and Methods

Study area and sampling

The sampling was done in Kermanshah area, west of Iran, in July 2021. Kermanshah has a hot-summer Mediterranean climate which is heavily influenced by the proximity of the Zagros Mountains. The annual precipitation average in Kermanshah is 149 mm with a maximum temperature of 45 °C and a minimum temperature of -10 °C. Blood samples were collected from a dog shelter for stray dogs located in the northern part of the city. Ninety-two mature dogs of both genders (30 males and 62 females) ranging from 1 to 8 years old were enrolled in this study. The age of the dogs was estimated according to dental abrasion and tartar. The age, sex, and clinical signs of each dog were recorded.

Hematological evaluations

A 5-ml blood sample was collected from the cephalic vein of each dog. Immediately, 1 ml of each sample was transferred to an EDTA-containing tube (K_3-EDTA, FL Medical, Italy) for complete blood count analysis. The rest of the sample was dispensed in a gel and clot activator-

containing tube (Vacumed®, FL Medical, Italy) for obtaining serum. Samples were transferred to the Diagnostic Laboratory of the Veterinary Hospital of Lorestan University in a cold container. All CBC analyses were performed in less than 18 hours from the time of sampling, also blood smears were prepared and stained with Giemsa on the same day.

The blood leukocyte count (WBC), erythrocyte count (RBC), Hemoglobin (Hb), mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count were determined using Celltac-alpha hematology analyzer (MEK-6550, Nihon Kohden, Japan). Differential leukocyte count was performed on Giemsa-stained blood smears.

Immunological evaluations

For immunological analyses, the blood collected in a coagulation tube was allowed to clot and then centrifuged at 2100× g for 15 minutes. Sera were collected and kept at -18°C for further evaluation.

Enzyme-linked immunosorbent assay (ELISA)

All 92 serum samples were evaluated for the presence of anti-*Leishmania infantum* antibodies using an indirect ELISA kit (ID Screen Leishmaniasis Indirect, ID.vet, France) according to the

manufacturer's instructions adopted from Mary *et al.* (1992). Briefly, 190 µl of buffer was transferred to the wells of the ELISA plate. Then, 10 µl of negative and positive controls and serum samples were added to the wells and incubated for 45±5 minutes at 37±2 °C. After that, wells were washed three times with the washing solution avoiding drying of wells between washing times. Next, 100 µl of 1x conjugate was added to all wells and they were further incubated for 30±3 minutes at 37±2 °C. The wells were washed again three times and then 100 µl of substrate solution was added to the wells and left for another 15±2 minutes at 21±5 °C. In the final step, 100 µl of stop solution was added to the wells and the absorbance optical density (OD) of wells was read using a microplate reader (ELx800, BioTek, USA) at the wavelength of 450 nm. The ratio of OD of each sample to mean OD of positive control was expressed as S/P values (%) according to the following formula. Samples with the S/P values ≥50% were considered as positive.

$$\frac{S}{P}(\%) = \frac{OD_{sample}}{OD_{positive\ control}} \times 100$$

Direct agglutination test (DAT)

For titration of anti-*Leishmania* antibodies, ELISA-positive samples were investigated with DAT according to Harith *et al.* (1989) procedure. The *L. infantum* antigen was prepared by the School

of Public Health, Tehran University of Medical Sciences. DAT antigens were made using mass production of promastigotes of *L. infantum* (MCAN/IR/07/Moheb-gh) in RPMI1640 plus 10% fetal bovine serum, trypsinization of the parasites, staining with Coomassie brilliant blue, and fixing with formaldehyde 1.2% (Harith *et al.*, 1989).

Previous studies showed cutoff points of 1:80 and 1:320 in asymptomatic and symptomatic dogs, respectively (Mohebali *et al.*, 2005). A two-fold dilution series of serum samples was made from 1:80 to end-point dilution of 1:20480 in a V-shaped microplate and incubated for 1 hour at 37 ºC. Fifty microliters of reconstituted DAT antigen was subsequently added to each well containing 50 µl of diluted serum. Quantitative results obtained with the DAT are expressed as an antibody titer, i.e. the reciprocal of the highest dilution at which agglutination (large diffuse blue mats) was still visible after 18-hour incubation at room temperature, compared with negative control wells, which had clear blue dots. The standard positive control serum was prepared from dog with *L. infantum* infection that was confirmed by culture and animal inoculation with 1:20480 titers (Harith *et al.*, 1989; Mohebali *et al.*, 2005).

Statistical analysis

The seroprevalence of CVL in stray dogs of Kermanshah was estimated from the ratio of results positive by the ELISA technique to the total number of dogs examined. Hematological

parameters of infected dogs were compared with normal ranges and alternations were summarized. Assessment of the associations between the seroprevalence with age and sex of the dogs were made with Chi-square and Fisher's exact tests. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). *P* values of ≤ 0.05 were considered statistically significant.

3. Results

From 92 serum samples that were examined by ELISA, 11 samples (11.95%) were positive. The anti-*Leishmania* antibody titration of seropositive samples was assessed by DAT (Table 1). Among seropositive dogs, 4 cases (36.36%) showed CVL clinical signs, including lymphadenomegaly and depilation (4 cases), pale mucus membranes and emaciation (3 cases), and skin ulcers (1 case). The remaining seropositive dogs were asymptomatic (Table 1).

Hematology results are shown in Table 2. The most alterations were observed with MCHC, band neutrophils, and lymphocytes. Two out of 11 infected cases (18.18%) have shown microcytosis. According to the hematocrit of infected dogs, 3 cases (27.27%) had anemia, while 2 cases (18.18%) had hemoconcentration. There was no association between DAT titers and the level of hematological abnormalities.

According to the serological results, the difference between the two genders and between the different age groups were not statistically significant (P>0.05; Table 3).

4. Discussion

Leishmania infantum is the main cause of Mediterranean VL in humans and reservoirs (Moradi-Asl *et al.*, 2020). The number of urban CVL cases is constantly increasing, and more domestic dogs and humans are at risk. The number of seropositive dogs from each zone should be considered an important risk factor of VL in humans (de Vasconcelos *et al.*, 2019). In the present study, 11.95% of the examined stray dogs from Kermanshah were seropositive for *L. infantum*. Similar seroprevalences of *L. infantum* were obtained from stray dogs of other provinces such as in Esfahan (10.8%) (Razzaghi Manesh *et al.*, 2012), in Kohgiluyeh and Boyer-Ahmad (10%) (Moshfe *et al.*, 2012), and in East Azerbaijan (9.1%) (Fallah *et al.*, 2011). On the other hand, higher seroprevalence levels were found in Ardebil (43.6%) (Taran *et al.*, 2007), in Mazandaran (34.6%) (Fakhar *et al.*, 2011), in Golestan (32%) (Fakhar *et al.*, 2014), and in Fars (30%) (Rassi *et al.*, 2007); while low values (from 2.6% to 4.98%) were observed in some other regions of the country (Khanmohammadi *et al.*, 2008; Malmasi *et al.*, 2014). These differences can be

attributed mainly to the weather and humidity conditions, geographical and seasonal distributions of vectors, and the serological methods applied for diagnosis.

The results of our CBC analyses showed alteration in some parameters. Most of our infected dogs had an increased number of band neutrophils (9 cases), which is indicative of inflammation in these animals. This result was not surprising as inflammation has been described as a frequent laboratory finding in CVL in other studies (Paltrinieri *et al.*, 2016). Most of our infected dogs had lymphocytosis (7 cases), while a few had lymphopenia (2 cases). Heidarpour *et al.* (2012) identified lymphocytosis in 21% of *Leishmania*-infected dogs in Mashhad area. They concluded that lymphocytosis is due to persistent antigenic stimulation from chronic *Leishmania* infection (Schultze, 2000). The lymphocytosis in our infected dogs may be indicative of chronic infection, but lymphopenia in two other positive cases may be due to stress response or acute inflammation (Meléndez-Lazo *et al.* 2018).

Eighteen percent of our infected dogs had low MCV which indicates microcytosis. This morphologic feature of RBCs may reflect impaired iron homeostasis that has been previously described in CVL. A reduction in transferrin concentration has been reported in leishmaniotic dogs, which was proposed to result in a reduction of iron (Silvestrini *et al.* 2014).

Although anemia has been described as a prominent laboratory finding in dogs suffering from leishmaniasis, in this study only 3 cases (27.27%) had anemia, while surprisingly 2 cases (18.18%) had hemoconcentration. The etiology of anemia in CVL is multifactorial, with anemia of chronic disease being likely the most important cause and hemorrhage, hemolysis, chronic renal failure, and bone marrow hypoplasia are other possible contributing causes (Meléndez-Lazo *et al.* 2018). In addition, the anemia is more frequent in dogs showing clinical signs than in subclinical seropositive dogs (Meléndez-Lazo *et al.* 2018). The presence of hemoconcentration in two cases could be a result of dehydration, respiratory insufficiency, or cardiovascular disease. Thrombocytopenia is considered a frequent laboratory result in canine leishmaniasis (Paltrinieri *et al.*, 2016), however, this was an uncommon finding in our study and was only identified in 2 cases (18.18%). Meléndez-Lazo *et al.* (2018) in Spain reported thrombocytopenia in 5.9% and thrombocytosis in 11.8% of client-owned dogs naturally infected with *L. infantum*. Heidarpour *et al.* (2012) in Mashhad, reported both thrombocytopenia and thrombocytosis in 5.26% of infected owned dogs, while the others (89.47%) had normal platelet count. These findings indicate that the frequency of thrombocytopenia in CVL may be dependent on other parameters including co-infection and nutritional conditions of infected dogs.

Our results revealed that the high number of asymptomatic stray dogs (63.63%), could be have an important role as reservoir host in this area. We found no significant association between seropositivity and age or sex. Some studies indicated that the infection rate was significantly higher in males and in old age. This can be because of more exposure in nature and more probability of contact by vectors (Mohebali *et al.*, 2005; Shokri *et al.*, 2017; Hosseini et al., 2022; Nouroozi Kouh *et al.*, 2023).

5. Conclusion

This study represents the first investigation on CVL seroprevalence in Kermanshah area, west of Iran. The high frequency of infection among examined dogs indicates that stray dogs from this area have an important role as reservoir of visceral leishmaniasis. It needs early screening and frequent surveillance of CVL, especially in stray dogs, to decrease disease incidence in the people.

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لیشمانیوز احشایی در سگ های بدون صاحب ناحیه کرمانشاه، ایران: شیوع سرمی و ارتباط آن با تغییرات بالینی و هماتولوژیک

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چکیده فارسی

زمینه مطالعه: لیشمانیوز احشایی (VL) مسئول مرگ و میر به ویژه در بین کودکان در کشورهای درحال توسعه است. سگ های بدون صاحب مخزن آلودگی به لیشمانیوز احشایی هستند. سگ های آلوده که بدون علامت هستند می توانند به عنوان منبع عفونت برای انسان عمل می کنند.

هدف: این مطالعه با هدف بررسی شیوع سرمی لیشمانیوز احشایی در سگ های بدون صاحب ناحیه کرمانشاه و همچنین ارزیابی تغییرات بالینی و خونی در سگ های آلوده به *لیشمانیا اینفانتوم* انجام شد.

روش کار: از 92 قلاده سگ بدون صاحب 1 تا 8 سال نمونه گیری شد. نمونه‌های سرمی از نظر وجود آنتی‌بادی‌های ضد *لیشمانیا اینفانتوم* با استفاده از روش الایزا (ELISA) مورد ارزیابی قرار گرفتند. تمامی نمونه های مثبت با استفاده از آزمون آگلوتیناسیون مستقیم (DAT) تیتر شدند.

نتایج: در مجموع 11 سگ (11/95٪) از سگ‌های مورد بررسی به *لیشمانیا اینفانتوم* آلوده بودند. از بین سگ های سرم مثبت، تنها 4 مورد (36/36٪) علائم بالینی را نشان دادند. از بین سگ های آلوده سه مورد دارای کم خونی و دو مورد دارای غلظت خون بودند. بر اساس شمارش سلول های خونی، بیشترین تغییرات در ارتباط با MCHC، باند نوتروفیل ها و لنفوسیت ها مشاهده شد.

نتیجه‌گیری نهایی: فراوانی بالای سگ های فاقد علامت نشان داد که این مخازن می بایستی به عنوان منبع اصلی آلودگی به VL در این ناحیه در نظر گرفته شوند. به منظورکاهش بروز بیماری در انسان، نظارت و پایش مکرر لیشمانیوز احشایی سگ سانان (CVL) به ویژه در سگ‌های بدون صاحب ضرورت دارد.

کلمات کلیدی: الایزا، سگهای بدون صاحب، CBC، علائم بالینی، *لیشمانیا اینفانتوم*

Table 1. Titration of seropositive dogs with the DAT according to clinical signs

No.	DAT titer	Clinical signs
1	1:160	-
2	1:160	-
3	1:160	-
4	1:160	-
5	1:320	-
6	1:640	-
7	1:640	+
8	1:2560	+
9	1:5120	-
10	1:20480	+
11	1:20480	+

+: Symptomatic, -: Asymptomatic

Table 2. Frequency of hematological alterations in dogs infected with *Leishmania infantum* (11 cases)

Parameter*(unit)	Number of dogs			Reference intervals (Latimer, 2011)
	High	Low	WRI✦	
WBC (×10^9/L)	5	0	6	5.0-14.1
Segmented Neutrophils (×10^9/L)	2	2	7	2.9-12.0
Band Neutrophils (×10^9/L)	9	-	2	0.0-0.45
Lymphocytes (×10^9/L)	7	2	2	0.4-2.9
Eosinophils (×10^9/L)	5	-	6	0.0-1.3
Monocytes (×10^9/L)	0	4	7	0.1-1.4
RBC (×10^{12}/L)	2	3	6	4.95-7.87
Hemoglobin (gr/L)	1	3	7	119-189
Hematocrit (L/L)	2	3	6	35-57
MCV (fL)	0	2	9	66-77
MCH (pg)	0	5	6	21.0-26.2

MCHC (%)	0	10	1	32.0-36.3
Platelets ($\times 10^9$/L)	0	2	9	211-621

*WBC: leukocyte count; RBC: erythrocyte count; Hb: Hemoglobin; MCV: mean cell volume; HCT: hematocrit; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

✦WRI: Within reference interval.

Table 3. Prevalence of *Leishmania infantum* infection in different sex and age groups of Kermanshah stray dogs

Risk factors	Results*	
	Negative	Positive
Gender		
Male	4/30 (13.33%)	26/30 (86.66%)
Female	7/62 (11.29%)	55/62 (88.70%)
Age		
<2 years	4/37 (10.81%)	33/37 (89.18%)
2–5 years	7/41 (17.07%)	34/41 (82.92%)
>5 years	0/14 (0%)	14/14 (100%)
Total	11/92 (11.95%)	81/92 (88.04%)

*For each group numbers indicated represent: the number of positive case/total number of cases (percentage of positive cases to total number of cases in the group).