Original Article

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

Ali Heydari[1], Hamidreza Shokrani[2*], Alireza Rocky[1]

1. Department of Clinical Sciences, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran.
2. Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran.

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ABSTRACT

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

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

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

Results: Eleven dogs (11.95%) were infected with *L. infantum*. Only four (36.36%) showed clinical signs among the seropositive dogs. Three infected patients had anemia, while two had hemoconcentration. According to the blood count, most alterations were observed in the mean corpuscular hemoglobin concentration (MCHC), band neutrophils, and lymphocytes.

Conclusion: The high frequency of asymptomatic dogs indicates that these reservoirs must be considered the principal source of VL infection in this area. Frequent surveillance and monitoring of canine VL (CVL) is critical to decrease the disease incidence in humans, especially in stray dogs.

Keywords: Complete blood count (CBC), Clinical signs, Enzyme-linked immunosorbent assay (ELISA), *Leishmania infantum*, Stray dogs

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*** Corresponding Author:**
Hamidreza Shokrani, Associate Professor.
Address: *Department of Pathobiology, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran.*
Phone: *+98 (66) 33120109*
E-mail: *shokrani.hm@lu.ac.ir*

Introduction

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

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

Currently, CVL is common, at least in some districts of more than half of the country's provinces (Razzaghi Manesh et al., 2012). The prevalence of CVL in Iran varies from 2.6% to 93.3% from endemic to non-endemic regions (Shokri et al., 2017). CVL is partly resistant to the routine therapeutic protocols used by people. Therefore, one of the best ways to prevent human infections is to identify and control infected stray dogs (Chiyo et al., 2023; de Castro et al., 2022). Although laboratory test abnormalities are usually nonspecific in CVL, they are crucial for diagnosis, disease staging, therapeutic monitoring, and determination of 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, 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 in Kermanshah Province, Iran, using ELISA and DAT, and to evaluate the hematological alterations in dogs naturally infected with *L. infantum*.

Materials and Methods

Study area and sampling

Sampling was performed in the Kermanshah area of western Iran in July 2021. Kermanshah has a hot summer Mediterranean climate, which is heavily influenced by the proximity of the Zagros Mountains. The average annual precipitation in Kermanshah Province 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 in the northern part of the city. Ninety-two mature dogs of both sexes (30 males and 62 females) ranging from 1 to 8 years of age were enrolled in this study. The age of the dogs was estimated according to dental abrasion and tartar. 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. One mL of each sample was immediately transferred to an EDTA-containing tube (K_3-EDTA, FL Medical, Italy) for complete blood count (CBC) analysis. The remaining sample was dispensed in a gel and clot activator-containing tube (Vacumed®, FL Medical, Italy) to obtain the serum. The samples were transferred to the Diagnostic Laboratory of the Veterinary Hospital of Lorestan University in a cold container. All CBC analyses were performed less than 18 h from sampling, and blood smears were prepared and stained with Giemsa on the same day.

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 a Celltac-alpha hematology analyzer (MEK-6550, Nihon Kohden, Japan). Differential leukocyte counts were performed using Giemsa-stained blood smears.

Immunological evaluations

For immunological analyses, 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.

ELISA

All 92 serum samples were evaluated for anti-*L. infantum* antibodies using an indirect ELISA kit (ID Screen Leishmaniasis Indirect, ID.vet, France) according to the manufacturer's instructions. Briefly, 190 µL of buffer was transferred to each well of an ELISA plate. Next, 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, the wells were washed three times with the washing solution to avoid drying the wells between washing times. Next, 100 µL of 1x conjugate was added to each well and incubated for 30±3 minutes at 37±2 °C. The wells were rewashed 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 well's optical density (OD) was read using a microplate reader (ELx800, BioTek, USA) at a wavelength of 450 nm. The ratio of the OD of each sample to the mean OD of the positive control was expressed as S/P values (%) according to the Equation 1:

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

Samples with S/P values ≥50% were considered positive.

DAT

ELISA-positive samples were investigated with DAT according to Harith et al. procedure for titration of anti-*Leishmania* antibodies. *L. infantum* antigen was prepared by the School of Public Health, Tehran University of Medical Sciences. DAT antigens were prepared by 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 one hour at 37 °C. Fifty microliters of the reconstituted DAT antigen was 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-h of incubation at room temperature, compared with negative control wells, which had clear blue dots. The standard positive control serum was prepared from dogs with *L. infantum* infection, which 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 in Kermanshah Province was estimated from the ratio of positive ELISA results to the total number of dogs examined. Hematological parameters of infected dogs were compared with normal ranges, and alterations were summarized. The relationship between seroprevalence, age, and sex of the dogs was assessed using chi-square and Fisher's exact tests. Statistical analyses were performed using GraphPad Prism, software, version 6 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set as $P\leq 0.05$.

Results

Of the 92 serum samples that were examined using ELISA, 11(11.95%) were positive. The anti-*Leishmania* antibody titration of seropositive samples was assessed by DAT (Table 1). Among seropositive dogs, 4(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 dogs were asymptomatic (Table 1).

Table 2 presents the hematological results. Most alterations were observed in MCHC, band neutrophils, and lymphocytes. Two of 11 infected cases (18.18%) showed microcytosis. According to the hematocrit of infected dogs, three cases (27.27%) had anemia, while two cases (18.18%) had hemoconcentration. No relationship was observed between DAT titer and hematological abnormalities.

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

Discussion

L. infantum is the main cause of Mediterranean VL in humans and reservoirs (Moradi-Asl et al., 2020). Urban CVL cases are constantly increasing, and more domestic dogs and humans are at risk. The number of seropositive dogs in each zone should be considered an important risk factor for VL in humans (de Vasconcelos et al., 2019). In the present study, 11.95% of examined stray dogs from Kermanshah were seropositive for *L. infantum*. Simi-

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.

lar seroprevalences of *L. infantum* were obtained from stray dogs in other provinces, such as Esfahan (10.8%) (Razzaghi Manesh et al., 2012), Kohgiluyeh and Boyer-Ahmad (10%) (Moshfe et al., 2012), and East Azerbaijan (9.1%) (Fallah et al., 2011). In contrast, higher seroprevalence levels were found in Ardebil (43.6%) (Taran et al., 2007), Mazandaran (34.6%) (Fakhar et al., 2011), Golestan (32%) (Fakhar et al., 2014), and Fars Province (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 serological methods applied for diagnosis.

The results of our CBC analyses showed alterations in some parameters. Most of our infected dogs had an increased number of band neutrophils (nine cases), 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 (seven cases), while a few had lymphopenia (two 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 infections (Schultze, 2000). Lymphocytosis in infected dogs may indicate chronic infection, but lymphopenia in two other positive cases may be due to a stress response or acute inflammation (Meléndez-Lazo et al., 2018).

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

Although anemia has been described as a prominent laboratory finding in dogs with leishmaniasis, in this study, only three cases (27.27%) had anemia, while two 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 these 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 which was identified in only two cases (18.18%). Meléndez-Lazo et al. (2018). reported thrombocytopenia

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

Parameter*(unit)	Number of Dogs			Reference Intervals (Latimer, 2011)
	High	Low	WRI#	
WBC (×10⁹/L)	5	0	6	5.0-14.1
Segmented Neutrophils (×10⁹/L)	2	2	7	2.9-12
Band Neutrophils (×10⁹/L)	9	-	2	0.0-0.45
Lymphocytes (×10⁹/L)	7	2	2	0.4-2.9
Eosinophils (×10⁹/L)	5	-	6	0.0-1.3
Monocytes (×10⁹/L)	0	4	7	0.1-1.4
RBC (×10¹²/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 (×10⁹/L)	0	2	9	211-621

Abbreviations: RBC: Erythrocyte count; Hb: Hemoglobin; MCV: Mean cell volume; HCT: Hematocrit; MCH: Mean cell hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

#WRI: Within reference interval, *WBC: Leukocyte count;

in 5.9% and thrombocytosis in 11.8% of client-owned dogs naturally infected with *L. infantum*. Heidarpour et al. (2012) in Mashhad City reported thrombocytopenia and thrombocytosis in 5.26% of infected dogs, while the others (89.47%) had normal platelet counts. These results indicated that the frequency of thrombocytopenia in CVL may depend on other parameters, including co-infection and the nutritional conditions of infected dogs.

Our results revealed that many asymptomatic stray dogs (63.63%) could play a crucial role as reservoir hosts in this area. We found no significant relationship between seropositivity and either age or sex. Some studies have

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

Risk Factors		No. (%)	
		Results*	
		Negative	Positive
Gender	Male (n=30)	4(13.33)	26(86.66)
	Female (n=62)	7(11.29)	55(88.7)
Age (y)	<2 (n=37)	4(10.81)	33(89.18)
	2–5 (n=41)	7(17.07)	34(82.92)
	>5 (n=14)	0(0)	14(100)
Total (n=92)		11(11.95)	81(88.04)

indicated that the infection rate was significantly higher in males and the elderly. This could be due to greater exposure in nature and a higher probability of contact by vectors (Mohebali et al., 2005; Shokri et al., 2017; Hosseini et al., 2022; Nouroozi Kouh et al., 2023).

Conclusion

This study represents the first investigation of CVL seroprevalence in the Kermanshah area in western Iran. The high frequency of infection among the examined dogs indicates that stray dogs from this area play a crucial role as reservoirs of VL. Early screening and frequent surveillance of CVL, especially in stray dogs, to decrease the disease incidence in humans.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Lorestan University, Khorramabad, Iran.

Funding

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Authors' contributions

Conceptualization, supervision, investigation, and writing: Hamidreza Shokrani; Methodology: Alireza Rocky; Data collection: Ali Heydari; Data analysis: Hamidreza Shokrani and Alireza Rocky; Funding acquisition and resources: Ali Heydari.

Conflict of interest

The authors declared no conflict of interest.

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