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Scientific Report

Leishmania infantum in an ownership dog (*Dogo Argentino*) with lymphadenopathy and extensive cutaneous lesions: Hematological, biochemical, microscopical and molecular findings

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Abstract

Background: *Leishmania* spp. are the cause of a common zoonotic illness. Dogs are the main reservoirs of the parasites, which play a considerable role in infecting humans and other hosts. **Case description:** A 2-year-old male dog with evident acute skin lesions and ulcerative nodules on the face was referred to a small animal hospital in Mazandaran province, Iran. In order to detect *Leishmania* parasites, the popliteal lymph node (LN) was sampled for the microscopical examination and the PCR reaction. Also, the hematological and biochemical parameters were measured. **Findings/treatment and outcome:** Light microscopy on the LN sample stained with Giemsa revealed Leishman bodies inside and outside of macrophages. Laboratory findings showed mild leukocytosis, lymphocytosis, neutrophilia, low hematocrit, hyperglobulinemia, hyperproteinemia, hypoalbuminemia, declined albumin/globulin ratio, and hyperglycemia. The PCR and sequencing results confirmed that *Leishmania infantum* was the causative agent. **Conclusion:** Cutaneous leishmaniasis due to *L. tropica* and *L. major* is prevalent in humans and dogs in Iran. In this report, a generalized skin disease was evident caused by *L. infantum*, a viscerotropic parasite species. In addition to cutaneous signs, leukocytosis and anemia concordance with the change in biochemical parameters supported a visceral invasion. Therefore, this report is significant as the cutaneous form of the disease may also imply a complicated visceral illness. In Mazandaran province, the visceral type has been rarely reported. As a result, this form of leishmaniasis in dogs raises concerns about the possibility of zoonotic transmission and may threaten public health.

Key words: Canine visceral leishmaniasis, Cutaneous disease, Iran, ITS-1, *Leishmania infantum*

Introduction

The zoonotic disease, known as leishmaniasis, is caused by prevalent species belonging to the genus *Leishmania* (Ikeda *et al*., 2008). The clinical disease is mostly caused by cutaneous and visceral invasions. Dogs and rats act as predominant reservoirs of cutaneous leishmaniasis (CL) (Mohebali *et al*., 2005), while the primary reservoirs of human visceral leishmaniasis (VL) are wild carnivores (Dantas-Torres, 2007).

There are several species of *Leishmania* that can infect dogs, including *infantum*, *tropica*, *major*, *braziliensis*, etc. (Reithinger and Davies, 1999; de Andrade *et al*., 2006). Of those species, the pathogenic potential of *L. infantum* has been more widely considered due to its systemic implications. The World Health Organization (WHO) reports fatalities in humans due to visceral leishmaniasis (VL) worldwide (Bi *et al*., 2018). In canids, *L. infantum* (from the *Donovani* complex) may also be characterized by cutaneous manifestations (Saridomichelakis and Koutinas, 2014). In the MiddleEast, the cutaneous form has mostly been associated with infection with two other species, *L. major* and *L. tropica* (Baneth *et al*., 2022; Fakhar *et al*., 2022). The latter species has sometimes been reported to be found in blood samples, implying its possibility of causing the visceral type. Also, cross-reactivity has been found between *L. tropica* and *L. infantum* (Hajjaran *et al*., 2007). Therefore, detection of the species involved should be concerned with predicting the pathogenesis of

the parasite.

The gold standard for laboratory diagnosis for *Leishmania* is the use of parasitological and serological methods. In addition, hematology, biochemistry, and clinical immunology are examples of nonspecific approaches (Ciaramella and Corona, 2003). Conventional PCR (CnPCR) and real-time PCR (qPCR) has undeniable advantages over traditional methods, including high sensitivity (Paiva-Cavalcanti *et al*., 2010; Mohammadiha *et al*., 2013).

In Iran, the *Leishmania* species have been described in dogs with an emphasis on the visceral type in recent years. The zoonotic aspect of visceral leishmaniasis in carnivores has remained significant in different parts of the country due to the reports on seroprevalence of *L. infantum* in human and canine samples (Mohebali *et al*., 2023). A serological routine, direct antiglobulin test (DAT), has been widely used to detect *L. infantum* infection in blood samples (Mohebali *et al*., 2006; Shokri *et al*., 2017) in many of epidemiological surveys. However, the serologic method maybe not valid to distinguish at the species level. Also, there is no documented information on the association between viscerotropic species and significant skin lesions in Iran. In this report, we describe the infection of a popliteal lymph node (LN) in a dog with considerable cutaneous lesions on the face. Also, the parasite was traced using the PCR method.

Materials and Methods

Ethics approval

The animal study and sample collection were reviewed and approved by the Research and Ethics Committee of the School of Veterinary Medicine, Shiraz University, Iran (No. MNN402/2024).

Case presentation and sample collection

An adult, 2-year-old male *Dogo argentino* was referred to a small animal hospital located in Mazandaran province, North of Iran. This region (53°6´ E, 36°23´ N) is geographically divided into two coastal and mountainous parts with humid weather and high annual mean rainfall (Fig. 1). The case had extensive skin lesions showing exfoliative dermatitis on the face, around his eyes, and on his nose (Figs. 2A-C). Ulcerative nodules were also observed in this area and some parts of the legs (Fig. 2D). The owner stated that these lesions had started to appear ten days before the visit. One month before the visit, the dog was lethargic and had lost his appetite. A physical examination revealed the presence of generalized eczematous and exfoliative dermatitis (erythroderma) and LN enlargement.

After a clinical examination, the popliteal LN was sampled using the fine needle aspiration technique, Giemsa stained, and underwent direct microscopical investigation. We also drew samples of serum and blood for biochemical and hematological analyses, respectively. Hematological factors were measured by an automated hematology analyzer (Nihon Kohden, MEK-6450 Celltac Alpha, Tokyo, Japan). Moreover, measurements of biochemical parameters were done using commercial kits supplied by Biorex Co. (Fars, Iran), and an AutoAnalyzer (Alpha® Classic, Iran).

Fig. 1: Map of the region where the infected dog was found and sampled

Fig. 2: A 2-year-old male *Dogo Argentino* showing extensive skin lesions with exfoliative dermatitis on the face (**A** to **C**), and also ulcerative nodule in this area (**D**)

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from the prepared LN smear using a commercial kit (Parstous®, Iran). The ribosomal DNA gene targeting the internal transcribed spacer 1 gene (ITS1) region was considered to detect *Leishmania* parasites using primers LSGITS1-F1: CAT TTT CCG ATG ATT ACA C and LSGITS1R1: CGT ATG TGA GCC GTT ATC (de Almeida *et al*., 2011). The amplification reaction involved a touchdown PCR program as follow: The initial denaturation performed at 95°C for 5 min, followed by 35 cycles including

denaturation at 94°C for 1 min, annealing at 58-54°C (the temperature was reduced 1°C in each subsequent cycle and fix the final annealing temperature at 54°C) for 1 min, extension 72°C for 45 s. The final extension step was done for 5 min at 72°C following the last cycle. Standard sequenced *L. infantum* specimen and distilled water were used as positive and negative controls, respectively. The results were electrophoresed on a 3% agarose gel and stained with Safe DNA Gel Stain (Sinaclone®; Iran). PCR products were sequenced (Pishgam Biotech Co., Iran), and the results were compared with other available sequences in the NCBI database using the BLAST search. The MEGA X software was applied to align the sequences and the phylogenetic tree was drawn using the maximum likelihood method (Kumar *et al*., 2018).

Results

Light microscopy on the Giemsa-stained LN smear revealed the parasite amastigotes, known as Leishman bodies, both inside and outside of the macrophages (Fig. 3). The laboratory report disclosed mild leukocytosis: (white blood cell (WBC) = 22.3 × 10^3/µL), (lymphocytosis = 5.6 × 10^3/µL), neutrophilia: (segmented neutrophils = 15.2 × 10^3/µL), a left shift (band neutrophil = 0.60 × 10^3/µL), Hematological findings also showed a low hematocrit; however, the thrombocyte counts were normal (Table 1). Also, biochemistry analysis indicated hypoalbuminemia (2.3 g/dL), decreased albumin to globulin (A/G) ratio (0.3) and hyperproteinemia (10.1 g/dL) as a result of hyperglobulinemia (7.9 g/dL). Furthermore, hyperglycemia and mild azotemia were also detected as serum biochemical abnormalities (Table 1).

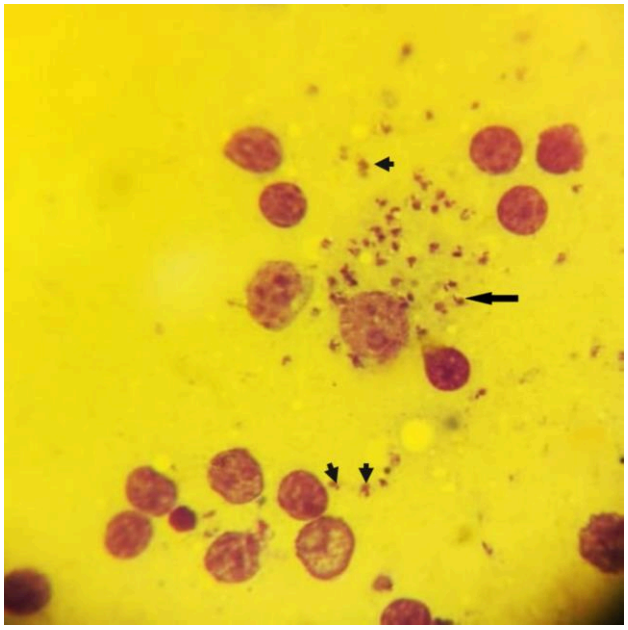

Fig. 3: Giemsa stained smear represented many of Leishman bodies (or amastigotes) of the parasite inside a macrophage cell (arrow) and freely in the field (arrow heads)

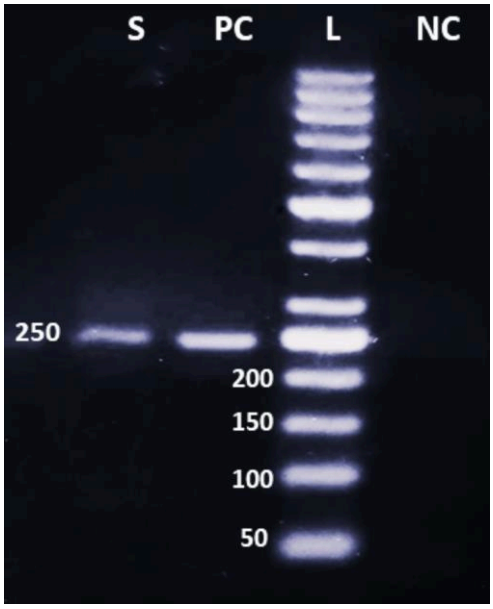

Fig. 4: The agarose gel shows the presence of the expected band of about 250 bp for the sample examined. Lane 1 (S): Sample, Lane 2 (PC): Positive control, Lane 3 (L): Ladder (Sina clone®, Iran; Cat No., SL7021), and Lane 4 (NC): Negative control

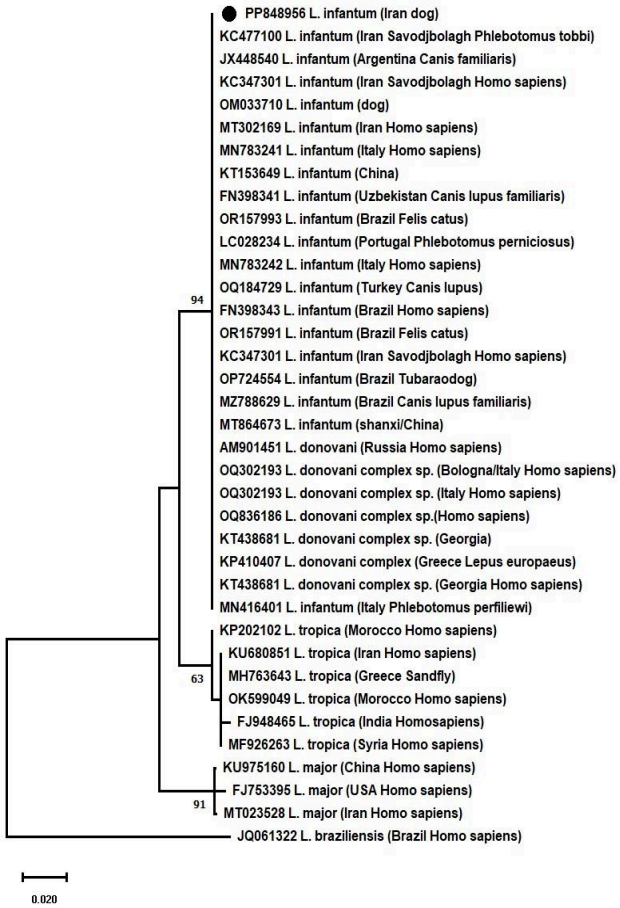

Fig. 5: Phylogenetic tree inferred from the analysis on the ITS-1 region using Maximum Likelihood method. The clades belonged to the species of *L. infantum* (including the present sample and the other from dogs, human and the sandflies), *L. tropica* and *L. major* are well seperated

Table 1: Values of the hematological and biochemical parameters in the *Leishmania* infected dog

Hematological parameters	Level*	Reference range	Biochemical parameters	Level*	Reference range
WBC ($\times10^3$ cells/µL)	22.3 H	6.0-17.0	Blood urea nitrogen (mg/dL)	37 H	10-35
Lymphocyte ($\times10^3$ cells/µL)	5.6 H	1.0-4.8	Urea (mg/dL)	79.2 H	15-70
Segmented neutrophil ($\times10^3$ cells/µL)	15.2 H	3.0-11.5	Creatinine (mg/dL)	2 H	0.5-1.7
Band neutrophil ($\times10^3$ cells/µL)	0.6 H	0-0.3	Triglycerid (mg/dL)	116	22-152
Monocyte ($\times10^3$ cells/µL)	0.20	0.15-1.35	Cholesterol (mg/dL)	151	95-337
Eosinophil ($\times10^3$ cells/µL)	0.70	0.10-1.25	Glucose (mg/dL)	153 H	70-120
HCT (%)	27 L	37-55	AST (IU/L)	60	10-66
RBC ($\times10^6$ cells/µL)	4.4 L	5.5-8.5	ALT (IU/L)	34	10-121
Hb (g/dL)	9.5	12-18	ALP (IU/L)	64	35-280
MCV (fl)	61	60-77	Calcium (mg/dL)	11.0	8.9-11.7
MCH (pg)	21.4	19.5-24.5	Phosphorus (mg/dL)	3.9	1.8-6.0
MCHC (%)	35.2	32-36	Albumin (g/dL)	2.3 L	2.5-4.5
Platelet ($\times10^3$ cells/µL)	577	200-500	Globulin (g/dL)	7.9 H	2.2-4.2
			Total bilirubin (mg/dL)	0.1	≤0.5
			Total protein (g/dL)	10.2 H	5.0-7.2
			A/G ratio	0.3 L	0.9-1.9

* H: High, and L: Low

In the molecular test based on the ITS-1 region, a band of about ~250 bp length was achieved (Fig. 4). According to the sequencing data and the BLAST search, the recovered specimen was *L. infantum.* According to the phylogenetic analysis, our specimen was grouped with a variety of *L. infantum* and *L. donovani* strains reported from dogs, human, sandflies and even cats (Fig. 5). These two species were separated well from *L. tropica* and *L. major*. The ITS-1 sequence data for the present strain was recorded in the GenBank under the accession number PP848956.

Discussion

In this study, we described a case infected with a visceral type of leishmaniasis showing a relatively widespread dermatitis, specifically on the face, which was accompanied by some hematological (anemia, leukocytosis, and neutrophilia with a left shift) and biochemical (hyperglobulinemia, hyperproteinemia, hyperglycemia, and mild azutemia) abnormalities.

The visceral infection has been frequently reported in seroepidemiological studies in Iran. This type of infection has more considered due to the zoonotic entity of the parasite (reviewed by Shokri *et al*., 2017; Mohebali *et al*., 2023). Dogs are one of the main reservoirs to transmit the parasite in human (Mohebali *et al*., 2005).

The first case of human visceral leishmaniasis (HVL) in Iran was reported by Pouya in 1949 from rural areas of Tonekabon, Mazandaran Province. This report also documented the first canine case in the same region (Pouya, 1949). While VL is endemic in several Iranian provinces, Mazandaran has historically been considered a sporadic area. This low incidence of VL in Mazandaran has been corroborated by local reports (Fakhar and Rahmati, 2011; Fakhar *et al*., 2011).

However, atypical and subclinical cases may go undetected due to the limited availability of specific diagnostic tools (e.g., DAT, PCR) and the potential for low clinical suspicion of VL among physicians in these regions. Our study underscores the importance of considering *L. infantum* in what has been considered a non-endemic area, thus emphasizing the need for improved diagnostic vigilance. The identification of *L. infantum* in a dog from the northern coastal provinces of Iran, a region previously considered non-endemic, highlights the need for updated epidemiological surveillance and raises concerns about potential misdiagnosis (Asgari *et al*., 2007). Consequently, strengthening molecular diagnostic capabilities and enhancing awareness among healthcare professionals in these regions are crucial for the early detection and effective management of the disease.

Based on previous investigations in Iran and the Middle East, the main causative agents of cutaneous leishmaniasis are *L. tropica* and *L. major* in dogs (Bamorovat *et al*., 2015; Baneth *et al*., 2016; 2017). On the other hand, cases of visceral infection with *L. tropica* have also been reported in dogs in Iran (Mohebali *et al*., 2005; Hajjaran *et al*., 2007). Another species, *L. infantum*, was reported from endemic regions for VL in Iran, but this parasite has usually not been associated with widespread cutaneous pathogenesis (Shokri *et al*., 2017). Therefore, our finding represents a rare case of canine leishmaniasis with extensive skin disease, which was confirmed by molecular and cytological methods on the LN sample.

From the hematological perspective, leukocytosis, neutrophilia, and left shift were seen in the presented case. The leukogram changes may not be as considerable in the symptomatic dogs as described by Ribeiro *et al*. (2013). But, in line with our data, other available research has linked the *Leishmania* infection to the observed WBC abnormality (Bogdan *et al*., 2001; Jafari Shourijeh *et al*., 2006). Nicolato *et al*. (2013) reported a high population of myelocytes, metamyelocytes, and band neutrophils among the granulocytic lineage precursors in the bone marrow. Based on the inflammatory response in multiple organs affected by parasitism, these results indicate a high demand for neutrophils in symptomatic dogs.

Anemia may be a common symptom of canine leishmaniasis, depending on the parasite load and pathogenesis. Typically, da Costa-Val *et al*. (2007) observed medullary hypoplasia and normocytic

normochromic anemia. Anemia tends to be related to the clinical stage (Dias *et al*., 2008). But in this instance, the hematocrit was measured at 27%, which was regarded as quite anemic. This finding, however, has been documented before and various factors have been indicated (Amusategui *et al*., 2003; Ribeiro *et al*., 2013). Serum protein analysis may detect anomalies at an early stage in the progression of the disease (Foglia Manzillo *et al*., 2013). Fitting with our results, there is a strong correlation between the severity of the clinical score and a rise in total proteins and globulins (Koutinas *et al*., 1999; Almeida *et al*., 2005; Solano-Gallego *et al*., 2009; Paltrinieri *et al*., 2010; Proverbio *et al*., 2014). Otherwise, the albumin-to-globulin (A/G) ratio drops for no valid reason other than that albumin is a negative acute phase protein and the proteinuric nephropathy can induce albumin loss as well (Almeida *et al*., 2005; Ribeiro *et al*., 2013). Some researchers have argued that a drop in the A/G ratio is the most sensitive indicator of canine leishmaniasis (Almeida *et al*., 2005; Geisweid *et al*., 2012; Paltrinieri *et al*., 2016). These outcomes are comparable to our biochemical findings. According to the literature, various types of samples have been used for molecular diagnosis. The PCR method on bone marrow proposed as a highly sensitive method to find visceral leishmaniasis (Oliva *et al*., 2006). However, in *L. infantum*-infected dogs, the popliteal LN and conjunctival sampling techniques are less invasive and result in good PCR conclusions (Marcelino *et al*., 2020). In this study, DNA obtained from Giemsa stained LN smear-as a minimally-invasive approach- was successfully amplified and sequenced using PCR assay.

This report describes a rare case of visceral leishmaniosis with generalized eczematous and exfoliative dermatitis (erythroderma) and LN invasion in a dog from a non-endemic area in northern Iran. The region has documented rare cases of visceral Leishmaniasis infection using serologic and molecular assays. Our study should be taken into account for infection control purposes, as *L. infantum* is a more dangerous species for canines and may threaten public health in this region of Iran.

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Conflict of interest

The authors declare no competing interests.

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